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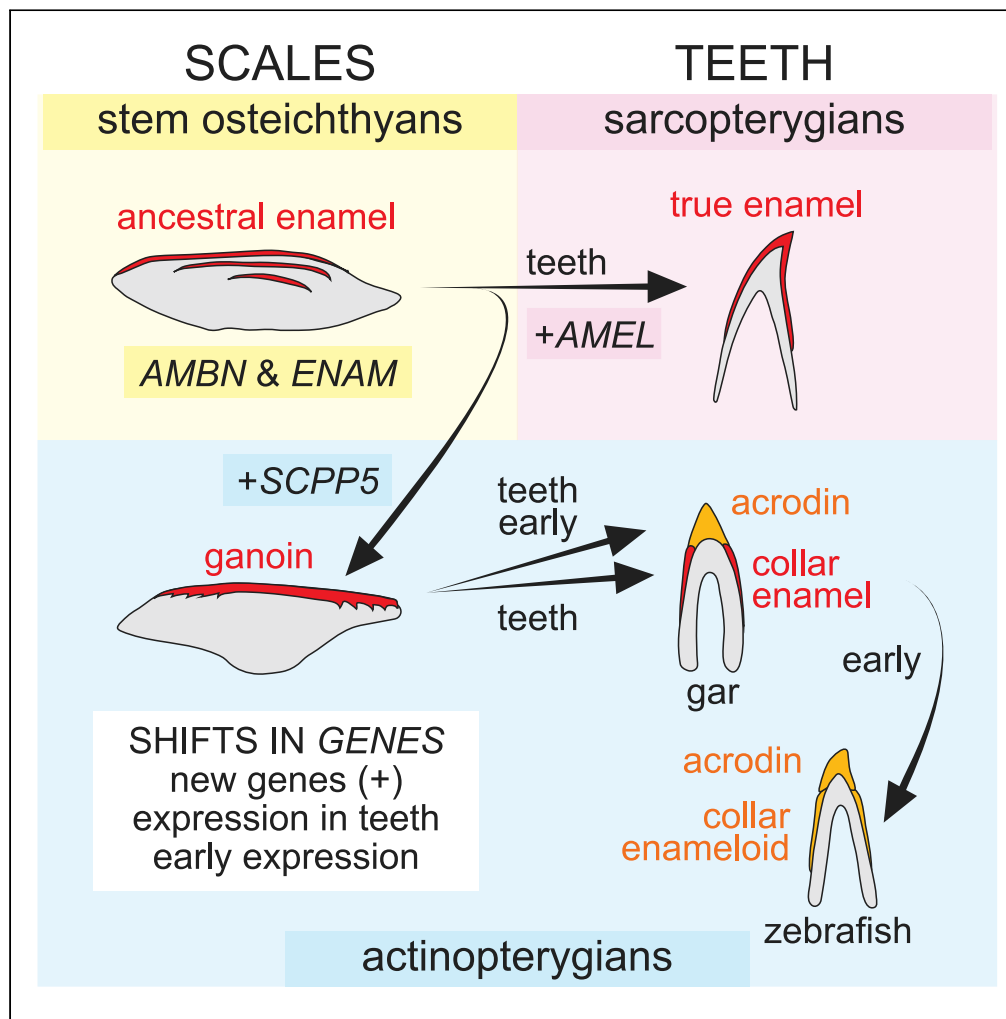
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Article

Coevolution of enamel, ganoin, enameloid, and their matrix SSCP genes in osteichthyans



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HIGHLIGHTS

Ganoin emerged in
actinopterygians; true
enamel arose in
sarcopterygians

Dental enamel, acrodin,
and enameloid in
actinopterygians are
related to ganoin

SCPP5 evolved in
association with ganoin,
whereas AMEL evolved
with true enamel

Shifts in SSCP gene
expression explain the
evolution of
hypermineralized tissues

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Article

Coevolution of enamel, ganoin, enameloid, and their matrix SCPP genes in osteichthyans

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SUMMARY

We resolve debate over the evolution of vertebrate hypermineralized tissues through analyses of matrix protein-encoding secretory calcium-binding phosphoprotein (SCPP) genes and phylogenetic inference of hypermineralized tissues. Among these genes, *AMBN* and *ENAM* are found in both sarcopterygians and actinopterygians, whereas *AMEL* and *SCPP5* are found only in sarcopterygians and actinopterygians, respectively. Actinopterygian *AMBN*, *ENAM*, and *SCPP5* are expressed during the formation of hypermineralized tissues on scales and teeth: ganoin, acrodin, and collar enamel in gar, and acrodin and collar enameloid in zebrafish. Our phylogenetic analyses indicate the emergence of an ancestral enamel in stem-osteichthyans, whereas ganoin emerged in stem-actinopterygians and true enamel in stem-sarcopterygians. Thus, *AMBN* and *ENAM* originated in concert with ancestral enamel, *SCPP5* evolved in association with ganoin, and *AMEL* evolved with true enamel. Shifts in gene expression domain and timing explain the evolution of different hypermineralized tissues. We propose that hypermineralized tissues in osteichthyans coevolved with matrix SCPP genes.

INTRODUCTION

The vertebrate skeleton is composed principally of cartilage, bone, dentine, enamel, and enameloid (Donoghue et al., 2006; Hall, 2015), all of which are critical to vertebrate adaptations, including protective body armor, an endoskeleton for locomotion, and teeth for feeding (Donoghue and Keating, 2014). As such, mineralized tissues comprise a key innovation underlying much of vertebrate evolutionary success. Among these skeletal tissues, the origin of hypermineralized tissues, enamel, enameloid, and their histological derivatives, remains controversial, partly because their classification in fossils varies among researchers (Friedman and Brazeau, 2010; Schultze, 2016) and partly because genes encoding their matrix proteins are well understood only for true enamel in sarcopterygians. Here we investigate the evolution of various hypermineralized tissues through genomic and developmental analyses, combined with estimation of ancestral states based on data available from living and fossil vertebrates. We draw these disparate approaches together to obtain a holistic understanding of the origin and diversification of vertebrate hypermineralized tissues. Our results reveal evidence for the coevolution of hypermineralized tissues in osteichthyans and their matrix secretory calcium-binding phosphoprotein (SCPP) genes.

RESULTS

Enamel and enameloid have been classified into different types depending on their location and histological characteristics (Figure 1A). Mineralization of enamel and enameloid progresses in organic matrices (Berkovitz and Shellis, 2016) that are subsequently removed as they mature into hypermineralized inorganic tissues (Sasagawa, 1997; Fincham et al., 1999). Enamel grows in a non-collagenous matrix secreted by ameloblasts of epithelial origin (Fincham et al., 1999) and occurs in three main types: (1) true enamel, considered equivalent to mammalian tooth enamel (Smith, 1989); (2) multilayered ganoin (Schultze, 2016) on scales and their derivatives, found only in bichirs and gars among extant clades, as well as in diverse fossil actinopterygians (Sire et al., 2009); and (3) tooth collar enamel, which occurs in actinopterygians, including bichirs, gars, and extinct clades (Smith, 1995; Ishiyama et al., 1999; Sasagawa et al., 2013). Enameloid forms in a collagenous matrix secreted by both inner dental epithelial (IDE) cells and mesenchyme-derived odontoblasts (Poole, 1967), often characterized histologically by protruding dentine tubules (Smith, 1995). Enameloid constitutes an acrodin tooth cap in various extant and extinct actinopterygians

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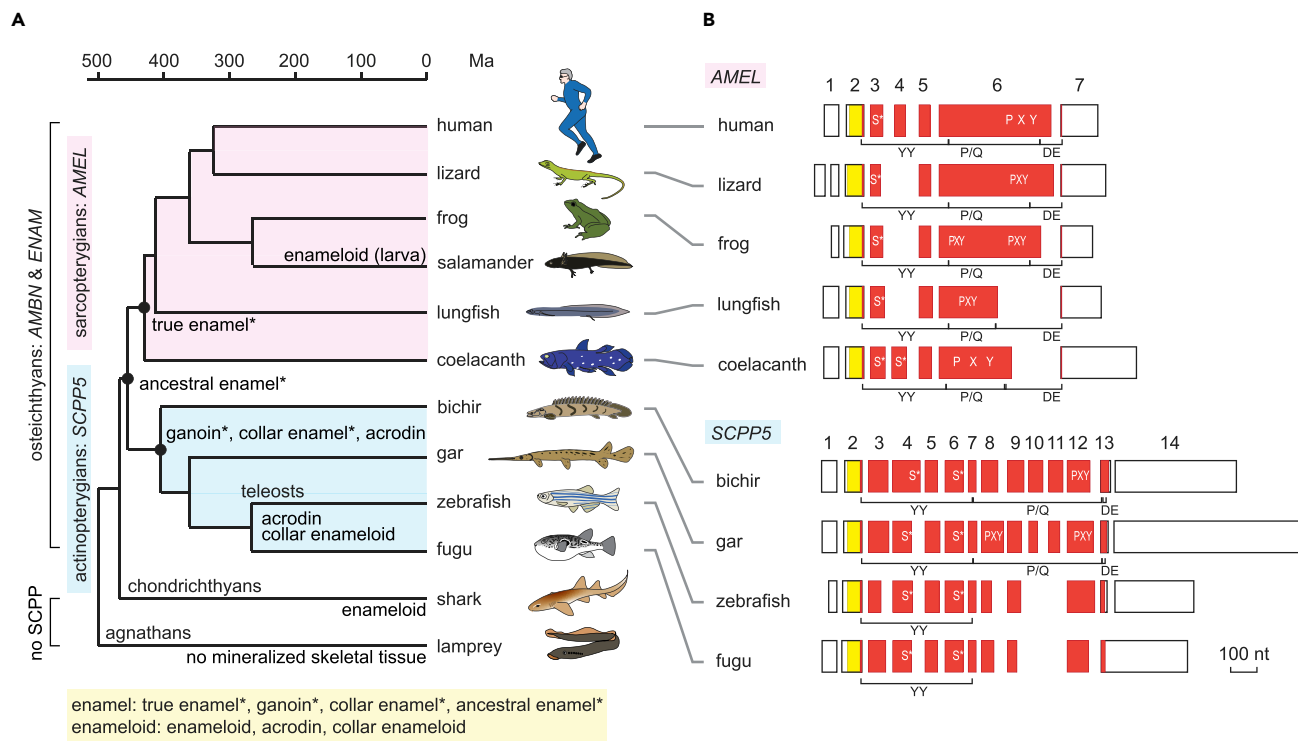


Figure 1. Phylogenetic distribution of enamel and enameloid matrix genes, and exon-intron structure of AMEL and SCPP5

(A) Phylogenetic distribution of enamel, enameloid, and their matrix genes. Hypermineralized tissues classified as enamel are denoted by an asterisk. Divergence times are based on previous studies: most sarcopterygian taxa (Hedges and Kumar, 2009), actinopterygian taxa (Near et al., 2012), lungfish-tetrapods and chondrichthyans-osteichthyans (Giles et al., 2017), and agnathans-gnathostomes (Kuraku and Kuratani, 2006).

(B) Exon-intron structure of AMEL and SCPP5. Separate boxes represent exons. Open areas in exons show 5' and 3' untranslated regions. The areas encoding the signal peptide and mature protein are shown in yellow and vermilion, respectively. Exon numbers are indicated for human AMEL (AMELX) and bichir scpp5. Locations encoding phospho-Ser residues (S*) (Kawasaki and Amemiya, 2014) and Pro-Xaa-Yaa (Xaa and Yaa represent any amino acids; PXY) repeats are illustrated within exon boxes. Regions encoding the three modules, the N-terminal aromatic residue-rich region (YY), the Pro/Gln-rich core region containing uninterrupted PXY repeats (P/Q), and the C-terminal hydrophilic region (DE), are indicated below exon boxes. Scale bar, 100 nucleotides (nt). See Figure S1A for details.

(Shellis and Miles, 1974; Sasagawa et al., 2013; Schultze, 2016). Tooth collar enameloid occurs in teleosts (Shellis and Miles, 1974; Sasagawa, 1988; Smith, 1995). Enameloid is also found on the dental and dermal skeleton in chondrichthyans (including acanthodians), as well as in extinct jawed and jawless stem-gnathostomes (Donoghue et al., 2006; Rücklin et al., 2011; Keating et al., 2015).

Enamel and enameloid matrix genes in sarcopterygians and actinopterygians

The amelogenin (AMEL), ameloblastin (AMBN), and enamelin (ENAM) genes encode the principal enamel matrix proteins (Fincham et al., 1999) in tetrapods (presumably also coelacanth [Kawasaki and Amemiya, 2014]), whereas SCPP5 is thought to encode an enameloid matrix protein in *Fugu rubripes* (fugu) and *Danio rerio* (zebrafish) (Kawasaki et al., 2005, 2009). AMEL has been found only in sarcopterygians and SCPP5 only in actinopterygians (Figure S1A) (Qu et al., 2015; Braasch et al., 2016; Kawasaki et al., 2017). AMBN and ENAM are also found in actinopterygians, including *Lepidosteus oculatus* (referred to below as "gar") and zebrafish (Figures S1B and S1C) (Braasch et al., 2016; Kawasaki et al., 2017). All four genes belong to the SCPP gene family that arose by gene duplication (Kawasaki and Weiss, 2003; Kawasaki et al., 2017). It is notable that no SCPP genes have been identified in chondrichthyans (Figure 1A) (Venkatesh et al., 2014; Enault et al., 2018).

We identified *amel* and *ambn* in *Lepidosiren paradoxa* (lungfish); *ambn*, *enam*, and *scpp5* in *Polypterus senegalus* (referred to below as "bichir") and various teleosts; and *ambn* in *Acipenser sinensis* (sturgeon; Figure S1); AMEL was not identified in actinopterygians. All three modules characteristic of tetrapod

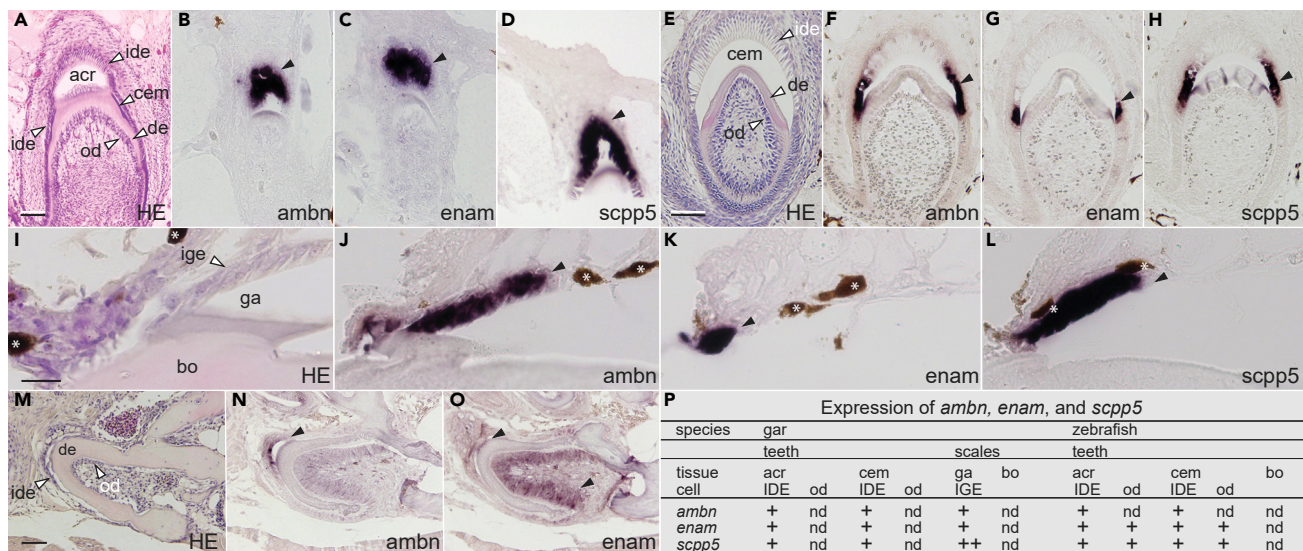


Figure 2. Expression of *ambn*, *enam*, and *scpp5* in teeth and scales of gar and teeth of zebrafish

(A) Hematoxylin-eosin (H&E) staining of a developing gar tooth immediately after acroдин formation. Scale bar, 50 μ m.

(B–D) Our *in situ* hybridization (ISH) analysis reveals expression of gar *ambn* (B), *enam* (C), and *scpp5* (D) in IDE cells during the matrix formation stage of acroдин formation (closed arrowheads).

(E) H&E staining of a developing gar tooth forming collar enamel. Scale bar, 50 μ m.

(F–H) ISH analysis reveals expression of gar *ambn* (F), *enam* (G), and *scpp5* (H) in IDE cells during the secretory stage of collar enamel formation (closed arrowheads).

(I) H&E staining of a gar scale forming ganoin. Ganoin was lost during decalcification. Scale bar, 50 μ m.

(J–L) ISH analysis reveals expression of gar *ambn* (J), *enam* (K), and *scpp5* (L) in IGE cells during the secretory stage of ganoin formation (closed arrowheads). Brown pigments are shown by asterisks.

(M) H&E staining of a developing zebrafish tooth. Scale bar, 50 μ m.

(N and O) ISH analysis reveals expression of zebrafish *ambn* (N) in IDE cells (a closed arrowhead) and *enam* (O) in matrix-formation stage IDE cells and odontoblasts (closed arrowheads).

(P) Summary of matrix SCPP gene expression in mineralized tissues. In gar scales, relative expression levels were determined by RNA-seq analysis (Table S1) and the highest expression level of *scpp5* among all SCPP genes is shown as “++.” Expression of zebrafish *scpp5* (Kawasaki et al., 2017) and stages of hypermineralized tissue formation in gar and teleosts were described previously (Sasagawa, 1995, 1997; Sasagawa and Ishiyama, 2005; Sasagawa et al., 2008, 2013). See Figure S2A for negative controls and more results. Abbreviations: acr, acroдин; bo, bone; cem, collar enamel; de, dentine; ga, ganoin; ide, inner dental epithelial cells; ige, inner ganoin epithelial cells; od, odontoblasts.

and coelacanth amelogenins (YY-, P/Q-, and DE-rich regions; Figures 1B and S1A) (Toyosawa et al., 1998; Fincham et al., 1999) were detected in proteins encoded by *amel* in *L. paradoxus* and *scpp5* in bichir and gar. Nevertheless, in most sarcopterygians these modules are encoded by five exons in *AMEL*, but twelve exons in bichir and gar *scpp5* genes (Figure 1B). Furthermore, *AMEL* differs from *SCPP5* in genomic location, exon-intron organization, and exons encoding phospho-Ser residues (Figure S1A). These data support interpretation independent origin of *AMEL* and *SCPP5*. Teleosts do not possess enamel, and their *scpp5* genes lack two of these three modules (P/Q and DE; Figures 1B and S1A).

Expression of *ambn*, *enam*, and *scpp5* in teeth and scales of gar and teeth of zebrafish

In gar, expression of *ambn*, *enam*, and *scpp5* was detected during tooth formation in IDE cells, initially during acroдин matrix formation (matrix formation stage of enameloid; Figures 2A–2D) and then during collar enamel matrix formation (secretory stage of enamel; Figures 2E–2H) (Sasagawa and Ishiyama, 2005; Sasagawa et al., 2008). During scale formation, expression of *ambn*, *enam*, and *scpp5* was detected in inner ganoin epithelial (IGE) cells that secrete the ganoin matrix on scales (secretory stage; Figures 2I–2L). We detected no other expression domains of these genes (Figure S2A). Given the limited expression domain of these genes in gar skin, their relative expression levels in IGE cells can be determined by RNA sequencing (RNA-seq) analysis of the skin (Qu et al., 2015; Braasch et al., 2016). Expression of *scpp5* was the highest among these genes and among all SCPP genes (Table S1).

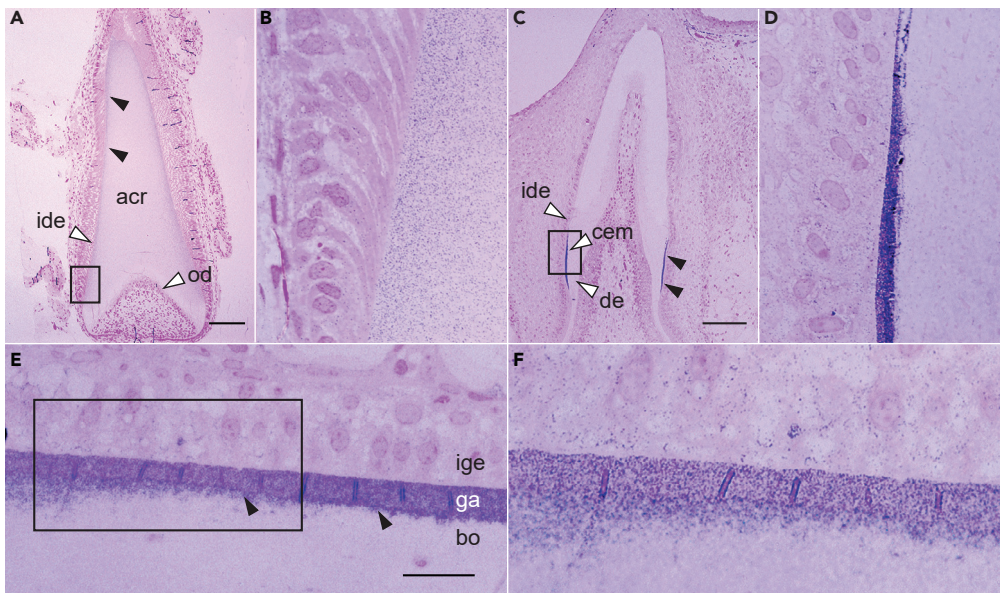


Figure 3. Distribution of gar Scpp5 in the matrix of teeth and scales detected using optical IHC analysis

(A–F) Optical IHC analysis of gar Scpp5 in acroдин (A and B) and collar enamel (C and D) in teeth, and ganoin in a scale (E and F). The rectangular region in (A), (C), and (E) is enlarged in (B), (D), and (F), respectively. IHC signals in (A), (C), and (E) are shown by closed arrowheads. Vertical lines in the ganoin layer (E and F) are artifacts of sectioning. See the legend of Figure 2 for abbreviations. Scale bar, 100 μ m (A and C) or 20 μ m (E). The contrast of these images was enhanced uniformly over the entire field (see Figure S2B for original images).

In zebrafish, expression of *ambn* and *enam* was detected in IDE cells in the matrix formation stages of acroдин and collar enameloid (Figures 2M–2O and S2A); *enam* was also expressed in odontoblasts, but we detected no significant expression of *enam* or *ambn* in bone cells (Figures 2N and 2O). Expression of *enam* in odontoblasts appears to initiate during acroдин matrix formation and continue throughout collar enameloid and dentine matrix formation (Figures 2O and S2A) (Shellis and Miles, 1974). Results of our expression analysis are summarized in Figure 2P.

Distribution of gar Scpp5 in acroдин and collar enamel on teeth and in ganoin on scales

The results of our *in situ* hybridization analysis for gar *scpp5* are consistent with the results of our immunohistochemical (IHC) analysis using an antibody raised against gar Scpp5. In the mineralization stage of acroдин formation, which begins after the matrix formation stage (Sasagawa and Ishiyama, 2005), high levels of Scpp5 were detected near the outer surface, decreasing toward the core, close to odontoblasts (Figures 3A and 3B). In the collar enamel matrix, Scpp5 was detected uniformly and strongly in the secretory stage, except in proximity to acroдин (Figures 3C and 3D), where Scpp5 was presumably absorbed by IDE cells that advanced to the maturation stage (Sasagawa et al., 2008). Scpp5 was also detected weakly in dentine along the border with collar enamel. In scales, Scpp5 was detected uniformly and strongly in the ganoin matrix in the secretory stage (Figure 3E) and underlying bone, decreasing immediately in a deeper region of the bone (Figure 3F).

Since our optical IHC analysis detected specific distributions of Scpp5 in the matrix of developing teeth and scales, we furthered IHC analysis using transmission electron microscopy (TEM). The results revealed an association of Scpp5 with the edge of collagen fibrils in the mineralization stage of acroдин formation (Figure 4A). In the developing collar enamel, Scpp5 was associated with electron-dense fibrils (Figure 4B) postulated to form as organic sheaths surrounding slender crystals (Warshawsky, 1989). Scpp5 was also detected in the underlying dentine near the border with collar enamel (Figure 4C), corroborating optical microscopic observations (Figure 3D). As in collar enamel, most Scpp5 signals in developing ganoin were associated with electron-dense fibrils (Figures 4D and 4E), especially along fibril edges, suggesting interactions of Scpp5 with minerals or mineral-associated organic molecules. Weak but significant signals

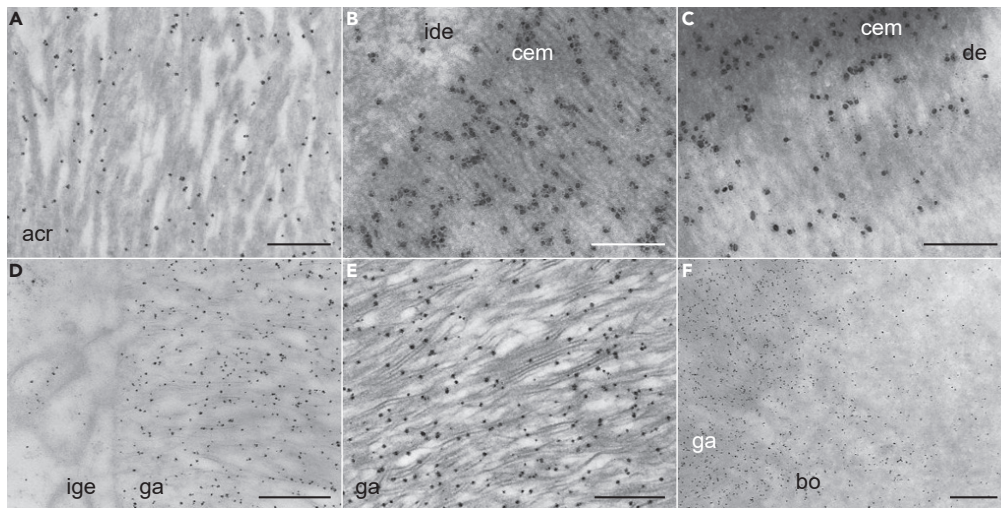


Figure 4. Distribution of gar Scpp5 in the matrix of teeth and scales detected using TEM IHC analysis

(A–F) TEM IHC analysis of gar Scpp5 in acroin (A), collar enamel near IDE cells (B), collar enamel near dentine (C), ganoin near IGE cells (D), a middle portion of ganoin (E), and ganoin near bone (F). Dark dots show the distribution of Scpp5. Scale bar, 500 nm (A, D, E, and F) or 200 nm (B and C). See the legend of Figure 2 for abbreviations and Figure S2C for negative controls.

were also detected in bone underlying scale ganoin, but not in deeper regions (Figure 4F), as in dentine underlying collar enamel (Figure 4C).

Reconstructing ancestral states of hypermineralized tissues

We explored the evolution of acroin, enameloid, enamel, and ganoin by reconstructing the ancestral states using data from living and fossil taxa (Table S2), stochastic character state mapping (Huelsenbeck et al., 2003), and phylogenetic hypotheses in which (1) *Guiyu*, *Achoania*, and *Psarolepis* (GAP clade) are constrained to stem-osteichthyans (King et al., 2017; Lu et al., 2017) and (2) these taxa are resolved as stem-sarcopterygians (Lu et al., 2016; Qiao et al., 2016; Choo et al., 2017) (Figures S3A and S3B).

Our results indicate that acroin evolved in the actinopterygian stem-lineage (*Cheirolepis* and more crown-ward taxa); its presence in *Ligulalepis* is a consequence of convergence or the isolated tooth from which these data derive does not belong to *Ligulalepis* (Figures 5A, 5B, S3C, and S3D). Dental enameloid evolved late in the chondrichthyan stem-lineage (*Pucapampella* or more crown-ward taxa; Figures 5A, 5B, S3E, and S3F); the presence of dental enameloid in acanthodians is convergent. Dermal enameloid was resolved as primitive to the chondrichthyan total-group and, although it is also present in ostracoderms (Donoghue and Sansom, 2002; Keating et al., 2015), it is inferred absent from the dermal skeleton of stem-osteichthyans (Figures 5C, 5D, S3G, and S3H). Thus, dermal enameloid was lost in placoderms (Giles et al., 2013) and evolved convergently in stem-chondrichthyans after the divergence of *Ramiro Suarezia* (Figures S3G and S3H). Taken together, these results suggest that, in both the dental and dermal skeletons, osteichthyan enameloid originated independently from chondrichthyan enameloid (but see below).

Our estimates of ancestral states were invariant to whether the GAP clade is resolved as stem-osteichthyan or stem-sarcopterygian (Figure 5). Dental enamel evolved early in the sarcopterygian stem-lineage (*Onychodus* and more crown-ward taxa), arising convergently as collar enamel in non-teleost actinopterygians (bichir and gar; Figures 5A, 5B, S3I, and S3J). Dermal enamel evolved deep in the osteichthyan stem-lineage (*Andreolepis* and more crown-ward taxa), was lost among early stem-actinopterygians (*Cheirolepis* and more crown-ward taxa), but retained in crown-sarcopterygians (Figures 5C, 5D, S3K, and S3L). Ganoin evolved among early stem-actinopterygians (*Cheirolepis* and more crown-ward taxa; Figures 5C, 5D, S3M, and S3N); the ganoin-like (Ørvig, 1978; Schultze, 2016) overlapping enamel in *Ligulalepis* (Schultze and Märss, 2004; Schultze, 2016) is resolved as convergent. If dermal enamel and ganoin are considered homologous, consistent with the expression patterns of *AMBN* and *ENAM* in both sarcopterygian enamel and gar ganoin, the ancestral tissue evolved deep in the osteichthyan-stem (*Andreolepis* and more crown-ward

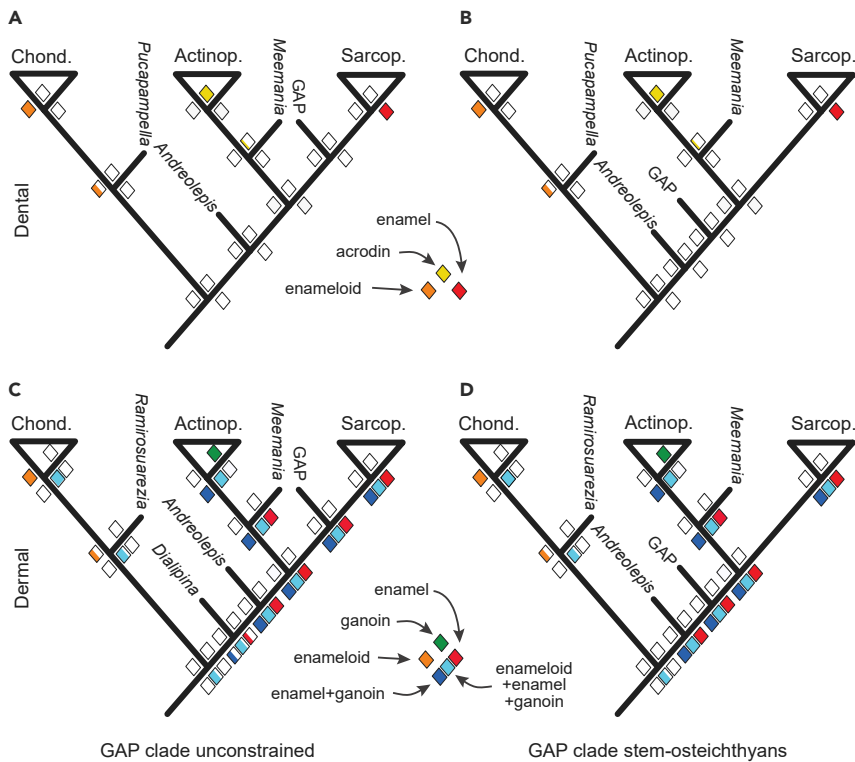


Figure 5. Reconstructing ancestral states of hypermineralized tissues

(A–D) Ancestral state reconstruction of the presence of hypermineralized tissues in the dental (A and B) and dermal (C and D) skeletons for trees, in which the GAP clade is resolved through unconstrained analysis to a stem-sarcopterygian affinity (A and C), or constrained to a stem-osteichthyan affinity (B and D). Diamonds at nodes express probability of the presence of a specific hypermineralized tissue, with the proportion of the color fill reflecting probability. The crown-chondrichthyans (Chond.), crown-actinopterygians (Actinop.), and crown-sarcopterygians (Sarcop.) are shown as triangles, and important stem taxa by bars (e.g., *Meemannia*). Tables S2 and S3 show data used to construct these trees.

taxa; Figures 5C, 5D, S3O, and S3P). Given the absence of SCPP genes in chondrichthyans, early enamel matrix SCPP genes likely evolved in concert with a prototypic enamel in stem-osteichthyans, from which true enamel and ganoin arose, respectively, in sarcopterygians and actinopterygians, as discussed below (Figure 1A).

Dermal enameloid is present in stem-gnathostomes, early members of placoderm plesia, and chondrichthyans (Giles et al., 2013). It has been hypothesized that enamel replaced enameloid through shifts in the timing of ameloblast and odontoblast activity (Smith, 1992, 1995). Ancestral state estimation provides support for this switch in a combined coding of dermal enamel, ganoin, and enameloid (Figures 5C, 5D, S3Q, and S3R). The initial enamel matrix SCPP gene may have originated in stem-gnathostomes and may have subsequently been lost in stem-chondrichthyans. As we discuss below, however, it is more likely that the enamel matrix SCPP genes are primitively absent from chondrichthyans and were never involved in dermal enameloid development. The origin of *AMBN* and *ENAM* in stem-osteichthyans may be invoked in the developmental evolution of enamel from an ancestral dermal enameloid (Donoghue and Sansom, 2002; Donoghue et al., 2006; Keating et al., 2015).

DISCUSSION

Similar formation of ganoin and collar enamel, and of acrodin and collar enameloid

We detected a similar expression pattern of *gar scpp5*, *ambn*, and *enam* in secretory-stage IGE cells and the uniform distribution of *gar Scpp5* in the ganoin matrix (Figures 2J–2L, 3E, and 3F), which suggests that all these three genes encode ganoin matrix proteins. During collar enamel formation, expression of *scpp5*, *ambn*, and *enam* in IDE cells (Figures 2F–2H) and the uniform distribution of *Scpp5* in the collar enamel

matrix (Figures 3C and 3D) were also detected. These results imply that ganoin and collar enamel form in a similar manner. Thus, collar enamel of gar is better described as collar ganoin, which corroborates the hypothesis obtained by histological studies of gars and many fossil actinopterygians (Ørvig, 1978; Schultze, 2016).

Acroдин forms below the basal lamina (BL) that originally separates odontoblasts from IDE cells (Smith, 1995). During acroдин formation in gar, odontoblasts retreat from the BL and secrete the bulk of the collagenous matrix (Sasagawa and Ishiyama, 2005), similar to dentine formation, while IDE cells secrete Scpp5, and presumably also Ambn and Enam, as suggested by the expression of *ambn* and *enam* in matrix formation-stage IDE cells. Thus, the region distal to the BL is formed principally by odontoblasts, whereas the contribution of IDE cells is large in the BL-proximal region (we observed the distribution of Scpp5 only near the outer surface; Figures 3A and 3B). This result suggests that gar acroдин forms as a dentine-ganoin composite, supporting the hypothesis for acroдин formation in teleosts (Shellis and Miles, 1974). Association of Scpp5 with collagen in the acroдин matrix during mineralization (Figure 4A) probably affects the mineralization and/or maturation processes, as previously inferred for teleosts (Shellis and Miles, 1974).

During acroдин formation in zebrafish, *ambn* and *enam* are expressed in IDE cells and *enam* are additionally expressed in odontoblasts. Similar to zebrafish *enam*, zebrafish and fugu *scpp5* genes are also expressed in both IDE cells and odontoblasts (Kawasaki et al., 2005; Kawasaki, 2009), unlike their gar orthologs (Figure 2P). Although the expression of these genes suggests conservation of acroдин in gar and zebrafish, expression of zebrafish *scpp5* and *enam* and fugu *scpp5* in odontoblasts implies a modification of acroдин. Because *ENAM* is expressed primarily in cells of epithelial origin during the formation of hypermineralized tissues in gar and sarcopterygians, the modification of acroдин is inferred to have occurred in teleosts. In zebrafish, a similar expression pattern of *ambn*, *enam*, and *scpp5* in IDE cells and odontoblasts was also detected during collar enameloid formation (Figure 2P) (Kawasaki, 2009). These results suggest that acroдин and collar enameloid form in a similar matrix in zebrafish and support the inference, obtained by studying various teleosts, that acroдин and collar enameloid are homologous, formed by both IDE cells and odontoblasts in a similar manner (Shellis and Miles, 1974; Sasagawa and Ishiyama, 1988).

Coevolution of hypermineralized tissues and SCPP genes

Our results suggest that enameloid evolved independently in chondrichthyans and actinopterygians. Although it is difficult to distinguish enameloid of chondrichthyans from enameloid of actinopterygians by histological characteristics (Reif, 1979), independent evolution of these tissues is supported by their different mineralization processes. In various actinopterygians, mineralization of enameloid initiates in matrix vesicles, and fine crystals accumulate along collagen fibrils, similar to mineralization of bone and dentine (Shellis and Miles, 1976; Sasagawa, 1988, 1997; Sasagawa et al., 2019). By contrast, mineralization of enameloid in various chondrichthyans begins in tubular vesicles, which are not found in osteichthyans, and no crystals concentrate along fibrillar structures (Sasagawa, 1998, 2002). The unique mineralization process of chondrichthyan enameloid reinforces the idea that this tissue evolved independently of SCPP genes (Kawasaki et al., 2017).

In gar, *scpp5* and presumably also *ambn* and *enam* encode ganoin matrix proteins, whereas *AMEL*, *AMBN*, and *ENAM* encode true enamel matrix proteins in sarcopterygians. The difference in matrix proteins of ganoin in gar and true enamel in sarcopterygians supports histological classification of true dental enamel in most stem- and crown-sarcopterygians and ganoin on scales in total-group actinopterygians (Qu et al., 2015). *AMEL* is found only in sarcopterygians; *SCPP5* only in actinopterygians (Figure S1). Gar *scpp5* shows the highest expression level among all SCPP genes in the skin during ganoin formation (Table S1), reminiscent of *AMEL* that encodes the most abundant enamel matrix protein (Fincham et al., 1999). Sarcopterygian *AMEL* genes and gar and bichir *scpp5* genes encode a similar modular structure, which is not encoded by *scpp5* in teleosts that do not possess enamel (Figure 1). Furthermore, expression of bichir *scpp5* was confirmed in the skin during ganoin formation (Figure S1A). These results suggest that sarcopterygian *AMEL* genes and gar and bichir *scpp5* genes have overlapping functions and that either *AMEL* or *scpp5* is sufficient for these overlapping functions during the formation of true enamel and ganoin. We thus assume that *AMEL* evolved in concert with true enamel and *SCPP5* evolved with ganoin.

Given the narrow expression domains and timings of *AMBN* and *ENAM*, similar spatiotemporal expression patterns of these genes during the formation of true enamel in sarcopterygians and ganoin in gar imply an

evolutionary relationship of these two tissues, rather than independent and coincidental employment of *AMBN* and *ENAM* in sarcopterygian true enamel and gar ganoin. Since sarcopterygians and gar phylogenetically bracket sarcopterygians and actinopterygians (Figure 1A) (Witmer, 1995), we hypothesize that both *AMBN* and *ENAM* were expressed during the formation of ancestral enamel (see below) in the most recent common ancestor of sarcopterygians and actinopterygians. The evolutionary relationship of true enamel and ganoin in gar and many fossil actinopterygians is supported by the common rod-like arrangement of crystallites, known as the protoprismatic microstructure (Ørvig, 1978; Smith, 1989; Sasagawa et al., 2016). A unique mineralization process of true enamel and ganoin in gar and bichir also supports their evolutionary relationship. During the formation of true enamel and ganoin, enamel ribbons form the mineralization front along the distal membrane of ameloblasts or IGE cells (Sire, 1995; Simmer et al., 2010). The close relationship of true enamel and ganoin suggests their homology: either one tissue derived from the other or both derived from an ancestral enamel.

Enamel and ganoin are considered homologous (Sire et al., 1987; Sasagawa et al., 2013; Qu et al., 2015), and the ancestral character estimation analysis assuming this (Figures 5C and 5D) is phylogenetically congruent, requiring an ancestral dermal hypermineralized tissue in stem-osteichthyans. Within the context of SCPP gene evolution, either *AMEL* or *SCPP5* was initially employed during the formation of an ancestral enamel in stem-osteichthyans; *SCPP5* was subsequently replaced by *AMEL* in sarcopterygians, or *AMEL* was replaced by *SCPP5* in actinopterygians. It is also conceivable, however, that *AMEL* arose in sarcopterygians and *SCPP5* in actinopterygians. Studies of gene-disrupted mice showed that both *AMBN* and *ENAM* are necessary for enamel ribbon formation, the unique mineralization process of enamel, whereas *AMEL* is not (Smith et al., 2016; Liang et al., 2019). Furthermore, a thin enamel can form in *AMEL*-deficient mice and in toothed whales that have deleterious mutations in *AMEL*, if both *AMBN* and *ENAM* are functional (Gibson et al., 2001; Kawasaki et al., 2020). These studies support the presence of ancestral enamel that formed enamel ribbons in a matrix containing *AMBN* and *ENAM*, but no *AMEL* or *SCPP5*. We hypothesize that both true enamel and ganoin originated from this ancestral enamel (Figures 1A and 5).

Dental acroдин arose with dermal ganoin among stem-actinopterygians. It was previously hypothesized that acroдин of teleosts is a composite of dentine and enamel (Shellis and Miles, 1974). Our observation of acroдин formation in gar confirms this hypothesis. Thus, acroдин evolution can be explained partly by early expression of ganoin matrix genes on the tooth cap during dentine formation, as previously suggested (Smith, 1992, 1995). Acroдин is similar in gar and zebrafish, but odontoblasts contribute more to acroдин formation in zebrafish by expressing *scpp5* and *enam*.

Ancestral state estimates suggest that dental enamel arose independently in stem-sarcopterygians and non-teleost actinopterygians. As we discussed above, collar enamel is better described as collar ganoin in gar and many fossil actinopterygians, which suggests that ganoin matrix gene expression on the tooth collar was critical to the evolution of collar enamel. Expression of *scpp5*, *ambn*, and *enam* during the formation of collar enamel in gar and collar enameloid in zebrafish suggests an evolutionary relationship of these two tissues and reinforces the previous hypothesis that the evolution of collar enameloid involved early expression of ganoin matrix genes during dentine formation (Smith, 1992, 1995). Consequently, collar enamel found in various non-teleost actinopterygians (Ørvig, 1978; Schultze, 2016) was replaced by collar enameloid in teleosts (Figure 1A). Enameloid is also found on teeth in larval urodeles (Assaraf-Weill et al., 2014; Berkovitz and Shellis, 2016), but in no other sarcopterygians (Figures S3C–S3F), indicating its independent origin in urodeles.

The results of our present study and previous studies using other methods and other species suggest that various hypermineralized tissues in modern osteichthyans can be classified genetically into true enamel, ganoin, and a diversity of enameloids; collar enamel, acroдин, and collar enameloid in actinopterygians are evolutionarily related to scale ganoin. A previous study supported the homology between ganoin and enamel largely based on the expression of gar *ambn* and *enam* in the skin (Qu et al., 2015); however, if acroдин and collar enameloid arose as a composite of dentine and ganoin, neither acroдин nor collar enameloid could be a ganoin homolog, even though *AMBN* and *ENAM* are expressed during formation. Our results show that orthologous SCPP gene expression may be insufficient to explain the differences between these tissues. The evolution of true enamel, ganoin, and enameloids can be better explained by various

changes of matrix SCPP genes, including spatiotemporal shifts in their expression. Hypermineralized tissues in osteichthyans appear to have coevolved with their matrix SCPP genes.

Limitations of the study

In extant actinopterygians, ganoin is found only in bichirs and gars. Although scales of bichirs consist of ganoin, dentine, and bone, scales of gars lack dentine. However, ganoin formation is similar in both clades (Sire, 1995), and bichir *ambn*, *enam*, and *scpp5* are presumably also expressed during scale and tooth formation in a manner similar to their gar orthologs. In teleosts, comprising ~30,000 species (Nelson et al., 2016), both acrodin and collar enameloid are found in various species. It was reported, however, that acrodin and collar enameloid of the common eel are covered with a hypermineralized layer formed by IDE cells, hence a type of enamel (Shellis and Miles, 1976). Although this layer remains to be confirmed, hypermineralized tissues may vary in some teleosts. Moreover, *ambn*, *enam*, and *scpp5* are all found in zebrafish and fugu (Figure S1), whereas *ambn* and/or *enam* were secondarily lost in some teleosts (Lv et al., 2017). The lack of these genes suggests modifications of acrodin and collar enameloid, or a loss of hypermineralized tissues. Examination of hypermineralized tissues in various teleosts would elucidate the evolution and adaptation of hypermineralized tissues in diverse teleosts.

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Kazuhiko Kawasaki (kuk2@psu.edu).

Materials availability

The anti-gar *Scpp5* antibody generated in this study is available from the Lead Contact with a completed Materials Transfer Agreement.

Data and code availability

The nucleotide sequences generated during this study are available at GenBank (accession numbers: MG010658-MG010662).

METHODS

All methods can be found in the accompanying [Transparent Methods supplemental file](#).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.isci.2020.102023>.

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AUTHOR CONTRIBUTIONS

Conceptualization, K.K., P.C.J.D., and M.I.; Investigation, all authors; Writing and Supervision, K.K. and P.C.J.D.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

Coevolution of enamel, ganoin, enameloid, and their matrix SCPP genes in osteichthyans

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A

	exon2	exon3	exon4	exon5
AMELX_human	MGTWILFACLLGAAFAM	LPPHPGHFG---YINF	NSHSQAINVDRTAL	VLTPLKWKYQS-IR---PP
AMEL_pig	MGTWIFFACLLGASLAMP	LPPHPGHFG---YINF	-----	VLTPLKWKYQNMIR---HP
AMEL_platypus	MGTWILFTCLIGAFAIP	LPPHPAHFG---YINF	-----	VLTPLKWKYQNMKR---QQ
AMEL_Anolis	MEGWTLVMCLLSTTFAIP	LPQ---HFG---YINF	-----	VMTPLKWKYQSLIG---HQ
AMEL_caiman	MEGWMLITCLLGATFAIP	LPPHPHHFG---YVNF	-----	VLTPLKWKYQSLMR---QP
AMEL_axolotl	MGPWILLTCLLSASCAMP	LPPHPHNFG---YINF	-----	VMTPLKWKYQSMMR---QQ
AMEL_Xenopus	MRLWVMTALTGAAFSVP	LPPHPHFG---YVNF	-----	ILSPLKWKYQSMMK---HQ
AMEL_lungfish	MKTLVLVFLACLLSSIAVP	LPPAGQH-GNFGVINI	-----	LLSPYAWLNKMMRQNP
AMEL_coelacanth	MKSLSLITCFLGAALALP	LGFPQQQCPGASIVNF	-----	MMSP-HWAD-WIRQQSQ

<- aromatic residue rich region (YY)

	exon2	exon3	exon4	exon5
SCPP5_medaka	MKFAILCLCLVSTACAVE	FQYLPHEF---GSRQQCP	GNNP-FSSGF-PQE	GLPGAYIEL IYPHRFPGGPTGGANET-Q
SCPP5_tilapia	MKLAILCLCLASTACAA	FQYLPHYT---GSRQQMP	MNNP-FTAGF-PHT	GLPGGYVEL IYPHRFPGGA-GVSSA-Q
SCPP5_fugu	MKLAILCLCLASTASAA	S---FFHYLPHYGGPQQVAP	VKTP-FTAAQTLTP	GLVGGYVEL LYPHRFPGTF--GANTA-Q
SCPP5_stickleback	MKLVIFFCLCLASTACAA	SI---FHYLPHYAGSRQQVE	VGNP-FTAGQSLPPP	GAAGAYVEL IYPHRVAGGV-GGTNAG-Q
SCPP5_zebrafish	MWTSLLCLLLAGAVSAA	LSFFNYLPHYGSPP	GNTGGFQGMSPQHP	AMNAPIIMEI IFPPRFPPANPAGGAAGT-S
SCPP5_eel	MKTAIACLCFASTICAA	MTSLYDYLPIGIVPPQPSQ	RTTNVVAFPAPPAQCP	GMAAPIIMEI VFPPRFPSQVTGGQGTG-S
SCPP5_gar	MKTIILLTCLFGSIFAAP	MQPLVEFLPOVETP-QCTAPYRQQPQQ	NNPYAPPSTPQQQ	GTPPAFPI LFPGYPRPSFGGPEHAGA
SCPP5_bichir	MKATVLLMCLLGTTFAVE	MORLYEFLPOVNSP-QQSAQPS-QPQP	SNPEAPIVPAOTPAAPPAEPAGPAPPALEI	MFPYGYPGQTLGQP---SS

<- aromatic residue rich region (YY)

	exon6
AMELX_human	YESYGYEPMGGWLHHIIPVLSQQHPPTHT-LQPHHHIPVVPAAQCPV-IPQQPMMFVPGQHSMTPIQHHQPNLP---P--
AMEL_pig	YTSYGYEPMGGWLHHIIPVVSQQTPQSHA-LQPHHHIPMVPAQCPG-IPQQPMMELPGQHSMTPIQHHQPNLP---L--
AMEL_platypus	YPSYGYEPMGGWLHHIIPVLSQQQTPTHA-LQPHHHIPVMAAQCPM-QPQQPMMFPGQPSVTPTOHHQSNLPQPAQQ--
AMEL_Anolis	YPRYGFEPMGGWMHHAAGP-TMHQTTFQSH-PSVHSTLHMQCPFHP---ALNPHMQPSGHNPFGSMFGQNTLMP-----
AMEL_Paleosuchus	YSSYGYEPMGGWLHQPMLP-IAQQHPPIQT-LTPHHQIPFLS-----PQHPLMQMPGPHQMMPIPOQQPSLOMPVQEPV
AMEL_axolotl	YPSYGYEPMGGWLQSQMMP-GSEPMFQQHM-----QPIPMQCPHHQMMLPQQPMLP---HGQMMPLPQHQQLLPQPGHQ--
AMEL_Xenopus	YPNYGYEPMSGWLQNPVVPAFPMMPQQCH---PSQHVVVKLPFHHHFLIPQQEVVEVP--HGVIELTPQHHTQLKPTYL--
AMEL_lungfish	YPAFGFEPMPW-----
AMEL_coelacanth	YFVIEIE-----VKRPPFMSPPFPISPMQPPQPMF-----

YY -><- P/Q-rich core region (P/Q)

	exon6	exon7	exon8	exon9
SCPP5_medaka	PFSSYGFIKYSIPQPPGRQLEI FYEYDFSQ-QR IVQNLPPMSNPIM-----PD VFP-IEYPPQNM-----PQQTIN			
SCPP5_tilapia	PFSSYGLIKYSIPQPPGRQVEV FYAYDFSQ-QR IIPNIPMTKGPV-----PE VLP-FDYPPQNI-----PQQNMN			
SCPP5_fugu	PFESHGFIKYSIPQPPGRQVEV YYPYDFSQ-QR ILTNIPPLTNVPHL-----PS VLP-FE-IPAH-----PN			
SCPP5_stickleback	SF---GFIKYSIPQPPGRQVEV YYPYDFSQ-QR IMTNLPPMTNSPQM-----PN VFP-FEYPPQNI-----PQQIPN			
SCPP5_zebrafish	SFFTQAFIKYSLPKAPGRKVEI FYPYDFGR-AQ DQPNVPLI---PQL-----PN IFP-FDLMPQTV-----PQQPPV			
SCPP5_eel	AFPSQAFIKYKIPKAPGRQVEI FYPYDLIQQQQ MLPNVPPMPAMPNIPQFPQI-----PN LFP-YGFLPPTG-----PQQN-N			
SCPP5_gar	MFPSSQAFIRQKIPQAGRLIEI LFPGYSGQHQQ MFPGASNMPPQPMQPPQLPQLPQQPN MFP-YGNMPPQTGG-----PQQNPN			
SCPP5_bichir	MFPSSQGFIRQKIPQPPGRQLEI LYPGYFGQ-QQ MFPGFSNLSPVPPMQ-----FPMPLN MFP-FGSMVPMM---PQQSPQTPN			

(YY)-><- P/Q-rich core region (P/Q)

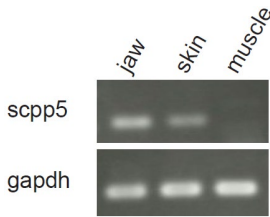
A (continued)



Chromosomal locations of AMEL and SCPP5.

	ARHGAP6	AMEL	P/Q-rich SCPP genes	SCPP5
<i>Homo sapiens</i>	X	X, Y	4	ND
<i>Anolis carolinensis</i>	3	3	2, 5	ND
<i>Xenopus tropicalis</i>	2	2	1	ND
<i>Latimeria chalumnae</i>	JH126997	JH126997	JH128739, JH128762, JH129486	ND
<i>Lepisosteus oculatus</i>	17	ND	2, 4, 22	4
<i>Danio rerio</i>	7	ND	1, 5, 10, 23	1
<i>Fugu rubripes</i>	1	ND	17	17

Expression of bichir *scpp5* in the jaw and skin, detected by RT-PCR.



B

	exon2	exon3	exon4
AMBN_human	---MKDLILILCLEMSFAVP	--FFFO-----	---SGTFGMAILLLE TMRQLGSL-QLNLTLSQ
AMBN_platypus	---MENLVLLSLFGTSFAIP	--VFLO-----	---PGGPPGMGILLLE TMRHLG---NLNLLFO
AMBN_caiman	---MNVWMLTLCLLGTGFALP	--MYFO-----	---HTGTRGMAMMLE -MROYGR--QNMNMLFO
AMBN_Anoelis	---MKQWMLVSSLLGICSAIP	--LFFO-----	---HAGIPGMGILLLE RTROLGGL-NIPPAVFO
AMBN_Xenopus	---MEPLVLVLCLLSTAMAYP	--MHFO-----	---APGTQGLAILLE TMRQQAANTLTAPFSSQ
AMBN_lungfish	---MKALSLLMCLLGTIHTLP	--LMFO-----	---QAGTFGVAILLE RMRKYGQ--FGMSFQDQ
AMBN_coelacanth	---MKRWILLGCFLGTAFSMP	--LMFO-----	---IPMARIVVLE KMRQYGG--SYMFOSSFO
AMBN_bichir	---MKAAILLICALGTTWAMP	MOVYQOMQOOIQLOOQFO-----	---HFGIPALATIQE MMRDIARYKSLMOHYPE
AMBN_sturgeon	---MKAVVILMCLLGTTCVFP	LOVYHQIYQOMQOQIQAEQAOQFOFGMLRYPIILE	MMQDIARYKRKFQOQHPQ
AMBN_gar	---MRSATVLMCLIGTTLSMP	LQVYQOLQROMLOQIHHQOQFOKQOQAGNPRYFILLQ	TMREMARYKAFMQOQSM
AMBN_zebrafish	MTQMRLIVMIVCFLAGTSAPV	-----	-----
AMBN_medaka	---MIIILLLSCTILLVSAVP	-----	-----
AMBN_tilapia	---MIIITLLSCFTIVVSAVP	-----	-----
AMBN_fugu	---MIVPTLLSWFIIMASAAP	-----	-----

	exon5	
AMBN_human	YSRYGFGKSFNSLMMHGLL-----	PPHSSLPWMRPR--EHETQQ--
AMBN_platypus	FSRFGFGKCFNSLMMHGFL-----	PPHSNYPWLSPR--DQETQQ--
AMBN_caiman	YGRYDYGEPFNSVWLHGLL-----	PPHSSFPWLQQR-PQEHETQQ--
AMBN_Anoelis	ALPYSYADPFHSMWGRSFL-----	PSHTFLWMTQGGPQRQDTQQ--
AMBN_Xenopus	ISRFQYNDPVSVLWLHGLL-----	PPHSSYPWLHOR-PQLSDNQQ--
AMBN_lungfish	FFVNGYNQFQSLLPWYQQL-----	QHLFPALVPWAOQP--HQENQQ--
AMBN_coelacanth	NPFWGYNPFVTSOQPDMSWL-----	---THLVNSMFPOLRLYPWLQPP--SCHDNQQ--
AMBN_bichir	LFHQLNFPDLQPGAPMNEAQQLEFQVPHGQAPSMPLPFOQFSQVFSQOLPFPOL-----	PPQLESVL--TPPLNFPOLPPQMPPHFQLIE-AQPGWEQQ--
AMBN_sturgeon	MFPSLNLPSAQPLPFPKKTGOQPPMQPQSQSPSLRFPQPPQFPFPFPFPQ-----	YQPPFPFPQSSFPOLFPNFQVVEYLPGLQOE-RLINRNQQ--
AMBN_gar	MFPSLNMPPHSTGLQPPQIPQFTGVQLPHNTGLQPPQIAQPSQTPTSCAP-----	SQLPFPQPSQOLPHQMTPSFPQVVEYQPGLOOM-QLVHAEQQ--
AMBN_zebrafish	ISPNA DLKESLSVG--DMQQ-----	FQPGSPRMLSGLHQOVENFNWASQPNF--STAVRQHEAQF--QYLEYSQQFPQ
AMBN_medaka	VAPNTHARMLPQGG--TORIHPVQSTNHKPEVQTPAFLSPIQEQAQSGIQPQDPQAVLEPSFSLFOHTWYPOGGSTINEMFQNVVFFLPANHLTLGQLPL	
AMBN_tilapia	VNSNVRRPILLQGGETATQGAQHVVAAANKPESPTPASISPSVQFO-AGGLQQDPQPI---	PSLQQYFWYPOGGMPMQ-----PLFYGPHQLTLPOOPL
AMBN_fugu	TGFSVHFSVIIQG-E-ATQGTDNEDYHTFAPFSSQVQCFALSLOPDPGRGGFHLI-----	SE-QFLWSFVGGAPTF-----VLLQOP-

	exon6	
AMBN_human	YEYSLEVVHPPPLPS-QESLKPQCPGLKPFLOS--AAATTNQAT-ALKEALQPFPIHLGHLFLOEGKLELVQQRVAPSDKPPKPE-----	
AMBN_platypus	YEYALFVVHPPPPRPS-QRTLOPQCPKQNAFLPF--TVAFSAQGPFAQNGDPOPPIILHLOPPLQOQEKPSVQEQVAFPKRPPKPE-----	
AMBN_caiman	YEYAMPVVHPPPLPSLOTPLPQCPRLQAQNP SLRSTLP TKQGQIQLN EALLP-VQVGQPPLOQGELFGIQQQQLIPADKOL-C-----	
AMBN_Anoelis	YEYAMPVVHPPPLPSLOTPIQFAEPRLPEQEFFQITNLIPTROVQIERYAIQPSIQQTFFQOQVEAHPPVDFQOQQLPAEROTHQ-----	
AMBN_Xenopus	FEYALPIHPPLPGAQSPAQTEKPGQHAQNMPE---QQPQAAASTDVSHQPSLPLGFPILQCADPAMMPKGGPADGQI-QTVALYM-----	
AMBN_lungfish	YEYALFVVHPPQPPQPHLPQCPPEVSPQOQPT-----	DQGQPSAQPSQOQHSFPQ-----
AMBN_coelacanth	YEYTLPIHPVQCP-QQPPAQPCQPPVQPIPT-----	QKTEQOQGCPEVQSPVQCGHQPPLQLDQOL-----
AMBN_bichir	YEYDAPVLLAHGPDQGEALPTAGNVVEVATPQAFVVEGQCVQVQCGGTQLNQPGQTPSAQGHLEVPVPGQCPQMFTTFGEVPIPEQT---AKQOQ	
AMBN_sturgeon	YEYESPVLFHQVPPQOSTAVPATPQASVQPAQDQCPGGRGDQPEHQIPIFNAPGQCPSHQ-----	GQCPQIFPTFGFLPMHHYQPVQCGQTPQ
AMBN_gar	YEYETEPFLLEQVPEGQCTELQVGATAGSAPATPTAPAAPROQGDQAGQCHETAQAPQPGVQVLQENIQIPQTGINGPQP---GTQGHQPTLPAQLPP	
AMBN_zebrafish	-----	-----
AMBN_medaka	-----	-----
AMBN_tilapia	-----	-----
AMBN_fugu	-----	-----

B (continued)

	exon7	exon8	exon9	exon10
AMBN_human	LPRVDFADPQGFS	LPQMDPDPDQGPS	LPGLDFADPQGST	IFQIAR-LISHG-----PMPQN--KQSP
AMBN_platypus	LPALDFTVQLROS	-----	-----	IFPIAQLISOG-----PROKA--EQSP
AMBN_caiman	LPALVYSGHLGOV	-----	-----	MYFIVHQLVHOG-----PMQBP--QCPA
AMBN_Anolis	-----	-----	-----	IYPALH-GTQOD-----PSQQL--QQQS
AMBN_Xenopus	-----	-----	-----	YQTIMNKLQOGAGETIIPD-PAGTLEVNQQH
AMBN_lungfish	-----	-----	-----	IEVEIF--QTFQVAQQP-QPFQQM--IFPA
AMBN_coelacanth	-----	-----	-----	VSPSNGNAVQTPQVTQQPLQPGQQG--QQPQ
AMBN_bichir	-----	-----	-----	-----
AMBN_sturgeon	-----	-----	-----	-----
AMBN_gar	-----	-----	-----	-----
AMBN_zebrafish	-----	-----	-----	-----
AMBN_medaka	-----	-----	-----	-----
AMBN_tilapia	-----	-----	-----	-----
AMBN_fugu	-----	-----	-----	-----

	exon11
AMBN_human	L--YPGMLYVPF-----GANQL-----
AMBN_platypus	L--YPELVYVPF-----GANQQ-----
AMBN_caiman	L--HPALFYMSY-----AANQG-----
AMBN_Anolis	I--YPSLYYMPY-----VANQG-----
AMBN_Xenopus	P--YPLGFFMQY-----GGGPG-----
AMBN_lungfish	F--E--YFYQF--FVQQGQOPM MFPFENSPP-QFPQQGQYFVMLFTSDILPFVIQQPDIQQPQLPQVPP TNSFLPSIVQQGGQQQQQQQQQQQQPQQ
AMBN_coelacanth	IQFFFTYEYLQMLPQVPGQQGQCPG GYPFYSFFP--ALACQPGLDL-----QQQQQQQQQQQQQQQQQQQQQQPQQQP
AMBN_bichir	-----FSGYGLLL-PASFPPQEV-----QEGLEQQPFQQQPSQS
AMBN_sturgeon	-----FPGYSFLI-PAAFPPQ-----QAQPGQEQQQQ
AMBN_gar	V--FNTF--GILPIMPPQAQDEQQ FPGYGVFF-NTAFPPQEV-----QPPQAAQPGAPFPQQQ
AMBN_zebrafish	F--F--YYPILGQQS---RNET FS-YGVSAFQHAASQQYPAANAALREARE-----QQGQNAHQMRQFPQQQQQQQQ
AMBN_medaka	M--FPPYGFYPLFAPPY---GNQM FSPYGFPSIRESFLOAPTQQLQNNQAPPAENVV-----PAGAAAPQQTQQ
AMBN_tilapia	I--FPPYGYLPLFSSPY---GNQL FSPYGYPMILESGLPQIPANQLNSFVLPAENAAGVAV-----DPSIDATQGIQQPQQQLQQQ
AMBN_fugu	I-SSPR-SFTPLISPAF---INQL-----

	exon12
AMBN_human	-----N-APARLGIMSTEEVA
AMBN_platypus	-----NAPARLGKVSSEEMF
AMBN_caiman	-----G-APARLGIVSSEEML
AMBN_Anolis	-----A-APARQGVISSEEMQ
AMBN_Xenopus	-----G-PPARLGAMSEET
AMBN_lungfish	GFPFIQFYLAALGHQ-NLPQN V-----QGAISSEELQ
AMBN_coelacanth	FPFSPFVYVPLPARQEQLPQG G-FPNTFGMSSEEMQ
AMBN_bichir	MFPOLVYVFLPLOT---GVMA G----AIGVSSSEEMQ
AMBN_sturgeon	MYPOLVYIQLOP---GMAG G----AIGVSSSEELQ
AMBN_gar	MIPQILYLQICPM--RSAMG G---GGFGASSEEMQ
AMBN_zebrafish	FQPIF-YMVEQMP-----QRM A---GSYGGLSSEELQ
AMBN_medaka	QNPPPIVYMLQ-----QPM N---PAIGGLSSEELE
AMBN_tilapia	N-PQILYMLQ-----QPM S---SELGLSSEELQ
AMBN_fugu	-----D---STLSFSEEEFE

B (continued)

exon13

AMBN_human GGR**E**-D**P**MAYGAM**F**PG**F**GGMR**P**-----G**F**EGM**P**HN**P**AMGGD**F**T**L**EF**D**S**F**VAATKG**P**EN-----E**E**GG**A**C-----GS**P**MP**E**AN**P**DNLEN**P**AF**L**TE**L**E**P**APHAG-LLV

AMBN_platypus GGGG-E**L**AYGS**I**F**P**G**F**GSMR**P**-----G**F**GAM**Q**IN**P**ALG**K**D**F**T**L**EDD**S**ETVAG**K**-P**P**G-----G**G**EV**L**-----GS**P**RDGA**I**PAG**H**EN**P**GL**L**PERISRLRSGAALT

AMBN_caiman GGRG-GV**F**AYGAM**I**P-----G**F**RGMP**Q**D**F**AL**Q**D**F**T**T**EDD**N**ATA**H**N-FA**I**-----G**G**AN**Q**G**F**SRGS**R**F**F**AVNRAGHGSA**I**LL**P**D**G**T**A**GH**E**G-----

AMBN_Anoelis GGG**F**-GAPAYRA**P**GP**D**L**F**AM**D**T-----R**F**GNS**P**LN-----G**P**GD**Y**T**V**EDD**T**L**G**IT**E**C**P**D**V**-----KGGANA-----GAN**P**SGKGNNAV**L**LD**G**T**Q**GGAL**P**-----

AMBN_Xenopus GGRTGA**A**HA**F**SS**L**Y**P**GL**L**GM**G**P-----R**L**ESH**Q**D**F**AL**Q**D**F**T**I**EDD**S**EVAG**Q**K-PT**G**-----G**E**VS**Q**G-----P**I**ES**P**Q**G**VGSS**I**MI**P**GLEGS**P**T**Q**-GET

AMBN_lungfish G**F**P**I**Q**F**Y**L**QALGH**Q**N**L**P**Q**N**V**Q**G**A**I**-E**E**I**Q**ANRAAGAAAA**A**V**F**NS**M**F**F**AF**A**AN**Y**P**F**GS**E**VAR**F**IS**V**DAT**I**EDD**H**EV**V**Q**G**E**Q**AG**Q**GTGT**P**GV**Q**N**L**PNAV**G**T-----P**S**SPAVN**I**P**I**EGN**P**T**G**

AMBN_coelacanth AAGG**G**LS-PALGG**I**IPGYVG**N**IP**G**-FGAG**V**PS**F**GGSV**P**GAAN**I**PM**F**-----G**F**PN**T**AV**N**PAG**Q**GGV**T**VEDD**P**PP**A**AE**K**-PAS**F**GN-SPGT-GAN**P**SG**Q**GGSA**P**EN**P**EGN**P**T**Q**VGS**I**P**N**P**Q**AN**P**PP**S**EG**T**PS

AMBN_bichir Q**S**AGDMS-P**E**MAG**F**IP**G**FAT**P**T---F**G**GG**F**PN**F**VSG**P**AVGG**T**VP**V**---AGGI---P**T**AG**T**Q**P**GV**L**EV-----K**L**E**I**P**V**

AMBN_sturgeon AAGG-**I**S-P**G**FAG**F**IP**G**FA**Q**A---F**G**GS**I**PS**F**GGSV**P**GA**E**GS**I**F---AGGV---P**T**Q**G**T**N**P-V**V**EA-----GL**P**T**V**E**I**LP**A**

AMBN_gar AAGG-VA---G**F**GG**F**V**P**GFAG**S**G---Y**G**GFGG**T**VP**G**T**E**---GV**F**PT**G**Q**I**L**F**VAPDAG**L**F-P**V**G**Q**GG-----S**P**TA**P**AV**F**PA

AMBN_zebrafish NMGR-VNMHL**P**AIRGN**V**F**V**SG**F**Q**T**V**P**IG**F**VR**P**PN**E**F**P**ATGMT**Y**PGIT**E**L**Q**Q**S**II**A**V**P**PSRD**A**I**P**ATGG**S**H-----P**S**NAV**L**P**S**RIS**G**T**Q**DRDRA**P**CV**E**A**E**I**P**LTNS**F**A**L**D---SGHG**A**F**I**SAD**V**E---

AMBN_medaka MQAK-L**N**QMS**V**---Y**S**VL**T**N**L**PAGAG**F**V**Q**EN**Q**AAG**L**T**N**P**G**Q**G**AT**P**HVGG**S**A-----AGVR**S**L**Q**GP**A**CSG**S**LLNS**N**SV**P**AGVK**T**AV**P**EANAV**Q**T**Q**N**H**A**K**

AMBN_tilapia MAAT-M**Q**QL**G**V---Y**M**P**N**VL**T**N**Q**PAGAV**Q**PES**Q**AAG**L**TH**P**E**Q**Q**G**V**Q**PAVG**T**SA-----AGA**Q**PI**K**AL**P**C-SGS**Q**P**S**ASG**S**AG-LAG**P**DAATA**Q**T**P**AE**P**Q

AMBN_fugu -----V**N**I---Y**L**STAL**T**D**S**AA**T**V**Q**V**K**Q**A**AG**L**-----Q**N**L**V**ST**V**GT**P**S-----TG**A**F**Q**N**Q**-V**T**SS**G**PP**M**DT**N**GV**F**V**G**-SE**K**P**A**EE**M**AT**V**CH**E**V**E**

exon13 (continued)

AMBN_human -----FL**K**DD**I**P**G**LPR**D**PSG**K**MG**L**P**S**VT**P**AA**V**D**P**LM**T**PE**L**AD**V**Y**R**T**Y**DAD**M**T**S**V**D**F**Q**EE**A**T**M**DT**M**A**P**NS**L**Q**T**SM**P**GN**K**A**Q**-----E**P**EM**M**HD**A**WH**F**Q**E**P

AMBN_platypus -----F**S**DF**I**PHRG**Q**GPAG**Q**SK**L**PP**G**VT**P**PP**A**D**P**LM**T**PG**E**SD**L**EFF**E**GD**G**T**T**PL**G**LQ**R**EAL**D**TT**M**T**P**DT**Q**ST**Y**MP**G**NE**A**Q-----Q**M**Q**V**T**Q**Q**Q**Q**F**Q**E**P

AMBN_caiman -----FL**P**NIND**M**PG**Q**GV**N**PVG**Q**-RG**T**PE**V**TP**T**ATA**P**EL**T**Q**G**IP**S**FM**T**F**G**AE**G**TV**P**LG**I**Q**K**EV**T**AD**P**TM**F**PE**A**Q**H**TL**M**AG**N**GA**E**-----Q**P**Q**M**Q**D**V**W**H**F**Q**E**P

AMBN_Anoelis -----NAN**S**HL**F**SPAG**Q**SG**L**SL**A**Q**A**T**A**PHAT**Q**G---P**L**PLDA**A**DE**S**-----M**P**LDA**T**MF**D**-LY**T**SN**V**GN**E**AG-----LA**Q**V**G**Q**D**A**W**H**F**Q**E**P

AMBN_Xenopus -----V**E**FP**N**INS**P**NMG**F**NE**Q**SKI**P**GP**T**E-AN**A**PR**L**THD**T**GAG**Y**VP**F**ALDD**T**MF**G**IG**Q**REN**V**IS**G**DI**T**RAN**N**AK**G**IS**E**IM**Q**H-----E**V**HL**Q**N**H**NN**Y**F**Q**E**P**

AMBN_lungfish Q**I**GN**P**Q**I**P-----Q**S**N**P**SS**P**GG**G**IM**Q**N**P**DIN**P**SN**Q**GL**P**AT**P**AD**I**PAAS**A**GN**P**SAAT**A**SP**N**EG**G**L-----P**V**I**Y**Y**E**GL**N**H**F**AA**R**GP**N**FP**P**

AMBN_coelacanth VOG**V**P**Q**PA**E**GN**L**P**N**LE**G**N-----P**T**G**Q**VGN**Q**PA**P**Q**S**K**G**PMGG**A**TP**A**ST**K**N**L**TP**A**N**V**PG**G**PEGG**I**VP**F**GAG**D**TASAA**A**R**K**V**V**PT**I**PA**F**FN**P**SLGG**T**AP**T**NN**N**AV**R**SN**A**AG**V**LP**P**IV**V**NE**G**LN-PLGG**G**W**I**Y**P**

AMBN_bichir GQ**G**-----GS**P**T**I**PE**V**IP**I**G**Q**GN**M**PK**T**EG**A**VP**A**GG**Q**Q**S**F**Q**M**K**LEN**M**T**P**D**G**V**L**LD**G**EV**P**PE**G**TV**Q**S**F**AP**G**ST**Q**D**T**IG**K**K**S**ET**A**AP**D**EA**T**VP-----KA**Q**AP**T**PE**E**V**E**MP**D**LD**D**Y**T**AN**F**Y**L**HP

AMBN_sturgeon GQ**G**-----GG**P**T**I**PE**V**IP**T**Q**G**SL**P**-----V**M**SK**T**GN**Q**PA**P**Q**S**K**L**PT**V**GL**T**TP**A**W**V**RL**T**PG**N**S**P**AAA.

AMBN_gar GHGN**L**FP**T**PG**S**-TV**P**V-----Q**G**S-----NN**Q**PA**P**Q**S**R**D**PA**F**SM**T**TP**A**GV**R**AAT**V**GN**P**AAAA**A**GT**A**Q**P**NAAAA**A**TAA**A**E-----RV**H**V**N**HA**E**R**N**I**P**AD**D**EM**P**EA**D**D**Y**RA**P**-FI**Y**P

AMBN_zebrafish -----TV**H**ED**E**Q**S**SG**E**N**L**VE**D**PLE**T**AE**Y**K**E**DL**F**EA**A**PP**E**-LLSD**T**VD**P**T**N**T**A**N-EM**Y**P

AMBN_medaka SL**P**TNR**N**LVR**S**K**A**N.

AMBN_tilapia LQ**P**MQ**E**TL**V**.

AMBN_fugu AE**L**LP**L**T**Q**TR**S**V.

C

ENAM_human
ENAM_pig
ENAM_platypus
ENAM_Anoelis
ENAM_Xenopus
ENAM_coelacanth
ENAM_bichir
ENAM_gar
ENAM_zebrafish
ENAM_trout
ENAM_fugu
ENAM_medaka
ENAM_tilapia

```

exon3      exon4      exon5
---MK-ILLVFLGLLGNSVAMP  MHMPRMPPGFSKKEE--  -----MMR-YNQFN--FMNGPH-
---MK-ILLVFLGLLCYSAAMP  MQMPRMPPGFSKKEE--  -----MMR-YGHFN--FMNAPH-
---MK-SFLLFLGLFGASTAMP  MHMPRMPPGFGKKEE--  -----MMF-YGOYN--FMNSPH-
MQRMK-QLLLYLCLIGTSWAVR  LRKPRKAGFGKKEE--  -----MMQ-YGPFQ--YLNSPQ-
---MMV-PIILLCLFISSNSVF  MHF---SHMSNKEE--  -----GME-YGYPG--YQN-PQ-
---MK-TLLLVCLLGTSLAGL  ARL---VRKARNKEE--  -----MVQKFTSYNRHMNQQL-
---MK-RGLLLLCFLGTALAF  V---GVALANQEE--  -----ALMQQ-RWNALLGAFQQQ-
---MK-IALLVLCFLGTSLAF  I---VPGAFIDHE--  -----AKMQQYRWYG-APQQQRQ-
---MK-AVALLMCLLGSSLAAP  -----APDQANE--  QATASHANTALQLMELYRMLGQLRQQ-
---MI-SVLLMCLLGFSLAAP  -----TPDQADE--  Q-VAAHANEALRWMELYRMYGSL---
---MVTILVALMVLGTAWSAP  -----ALGEEAEEN  --VAAHANQALRWMMHYRLY---QQQ-
---MK--LFVFLYLIISASAP  -----AEDDSE--  --VAAHANAAALRLMELFRMY---QQQ-
---MK-QLVFFICLLVSTLAAP  -----APAESE--  --IAAHANALRWMEMYRLY---QQQ-

```

ENAM_human
ENAM_pig
ENAM_platypus
ENAM_Anoelis
ENAM_Xenopus
ENAM_coelacanth
ENAM_bichir
ENAM_gar
ENAM_zebrafish
ENAM_trout
ENAM_fugu
ENAM_medaka
ENAM_tilapia

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exon6
M-AHL-GFFFGNG--LPQQFPQYQM--  -----PMWPQPPNT-WHPRKSS-----APKRHNKTD-
M-AHL-GTLYGNMQLPQFFFPQYQM--  -----PMWPQPPNK-KHPQKPS-----ASKQOSKTD-
M-AQL-GFLYGYGLQLPQFFFPQYQM--  -----PPWPQPPNG-WPAQNPQPPQ-----GGKPOAKPG-
L-TQLAASLYGYRSGFPQMVBPQQ--  -----PTFELQRFYL-WPQMPVHQSS-----ARPPQKAH-
M-PGI-GSMYGLG--FPHYSQMIQL-  -----QCAFGRQPS-SWLQNIARTQQIHKETIPLNLPRFHLPV-
QVMQL-RSLYGNAFQRL-MYBPQQQLQQQQQRKLLVARLLAQALKQTOQSRQQNAAPQLQVPEQLQWPLEEPQVQQ-----KQKQQQQLLQ
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ENAM_human
ENAM_pig
ENAM_platypus
ENAM_Anoelis
ENAM_Xenopus
ENAM_coelacanth
ENAM_bichir
ENAM_gar
ENAM_zebrafish
ENAM_trout
ENAM_fugu
ENAM_medaka
ENAM_tilapia

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exon6 (continued)
QTQETQKPNQ-----TQSKKPP-QKRFL-----KQPSHNOQFQPE-----EEAQPPQ-
PAPEQSKPNO-----POPKTPT-PKQPL-----NEPSPKTPTQPE-----EETQTPQ-
QSONTKQPNP-----SQPKPP-QQOPP-----KQAAKQPAQGPSKET-----EETROPO-
QTFVPEKKNSRERLKPRLVFPQPRYQQQPKKIHLPAKQPP-----VATHRPQPPQTFEQPPKGEKPOQPO-
QSLSQLPAP-----FVQQPPQLNPPQSKSNYVTKQPP-----HQLQLPPAPD-----QQAQAPM-
QAQNLKNVLP-----QQPQRLQQKPSQQPPQGLQHKKPFOQAKKQEQSRQR-----FSRKQPPQKDLQQL
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ENAM_human
ENAM_pig
ENAM_platypus
ENAM_Anoelis
ENAM_Xenopus
ENAM_coelacanth
ENAM_bichir
ENAM_gar
ENAM_zebrafish
ENAM_trout
ENAM_fugu
ENAM_medaka
ENAM_tilapia

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exon7      exon8
AFPPFGNGLFVYQPPWQI--PQ-----RLPPPQY-----GRPPIQNEE--GG
AFPPFGNGLFVYQPLWHV--PH-----RIPPQY-----GRPPTQNEE--GG
MYPPFGNGFFFPQLPWQI--PP-----QMPQY-----GRPPGQNEE--GG
AFPP-----HBPQWQF--PQIFGHGGEQPOLFNQYQ-----RMPF-----GRPPNNEE--G
MFPPFSNIMFVQBYW--QQ-----PFMHGF-----GRPPNNEE--GN
VYPSQGYIRMLPQMLWFQRCQQ-----PV-----GRRKQNGYEEQGMG
GNYF-----KVVYIQLPSPF-MAG-----MGPNR-----AAPAEQEE--GG
QDFP-----KVVYVHLPP--MAG-----MGPN-----AAPAEQEA--GA
GY-GA-----VAG--PQL-----P-----AAFAAVVDFAPVD  TQPVQVSPQVFPQ-----GFFNPAAFA-----PTGDGDEE--VA
GQ-LA-----ABA--QF-PMLN-----APAAAPATPAQDF-----KFFYPSPMN-----DEE--A
AMLQN-----PFV--FPAGRAQ-----VNAAPVQPLAVAPQAAVAHPAAVAHPAAVGDDEE--AQ
GLA-N-----PFL--PAAGAH-----INV-PVQPA-----AATPAGDADEE--AE
GIVQN-----PFV--PSANSF-----AVSAGAT-----VDAAPACPADVPE-----PAHVPEVGDAEE--TE

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C (continued)

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ENAM_human      exon9      -NPYFGY-----FGYHGFGG-RPPYYEEMFEQDFEKKKEEDPPKAESPTEPTANSTVTETNSTC-PNPK---GSCGGNDTSPTGNSTPG---L
ENAM_pig         -NPYFGF-----FGYHGFGG-RPPYYEEMFEQDFEKKKEKDPPKTEPTATEPSVNTTVETNSTC-PNAP---N-PRGNDTSPTGTSGCG---P
ENAM_platypus    -NPYFGY-----FGHQGGGG-RPPYYEEMKEEKEE-----SPKEDSPSEPTANSTVAETNSTC-PSGP-SQAGSCGGNATDFGQSAGNGSPAQ
ENAM_Anoelis     -NPYFGF-----GYQGMGG-RPPYYEEMFEQEDK-----EAPK-ESPAIDPVTNST--DTNSTV-SNP-----ASCGGNGTMSGLSEKATGANS
ENAM_Xenopus     -GNYFGF-----FGMGG-RAPYNEEINDEDEEPAKAESKTESPVAE-TSNTTIDNNSTT-ISQ-----SQGNATTNNESMAGDITQS
ENAM_coelacanth  -ISYFGA-----YYPEI-RRRPLEEEMTPESEG-----KSKKTD-----NSEVPEVSSFTSASP-----AGQDGNSTNAGGNHTVHGENL
ENAM_bichir      QPQYAGYMPPAYGYGYPYGGYGHFKRMPL--EEINEEGPAKAEKGKPEKPPKAEATPTPGGTATKPDGAAPNTEGGAPAAAGGSPAAEGSPNGQGGT
ENAM_gar         -POYGYVPSFYGYGFPFG-FGNFGRRAPL--EEINEVNEKESSEPGKPEAPAAATEAPKAEDLPPTFGGV--PTNTEGAGAAASANGTAANGAGNQPALEG
ENAM_zebrafish   -POYRGF-----YFHYGYHQAQAPAAPLNDEAEGAEAEAGAEAGAEAGAEAGAEVGAEE--PAGTAATVDAAAANEI---LPVDVAEPAA
ENAM_trout       -FVPRYGGYGRY-YFYPGLAAPAAPAVPLNDEVGEDEAAT-EAEAE-----PAVDHAAEEPAVVDTAAPVDPAD-AAFVDPAMDVPIDVVI
ENAM_fugu        -MQ-----AAGFGAPAAPLNDEAEEASEAEPAADPEAAGAAADATPAPAGA--EVAATAAADAPADAAAASGPEAAAADVPAAD-DVPA
ENAM_medaka      -DGNB-----GEGVGAVPLNDEEEVEVEVEATEVE-----PAVVETAATAAPFAAAAEPAVVDDVAVDAAPVDDVV-VDVPP
ENAM_tilapia     -DGNB-----ATKAAGPAPLNDEVEEAEEVEAAEAD-----PAMVEAEPSANPAAVVEPAVVDPIDV---APVDDAAADVVP

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ENAM_human      exon9 (continued) NRTGNPNSARNGI---GPLPAVNASGQGGPGSQIPW--RPSQPNIRENHYPYNI-RNFPSGRQWYF-----TGTVMGHRQNRPFYRNQQVQRG
ENAM_pig         NPSRNPQTQNG---PAVNVSGQGVPRSQSPW--GPRQTIHENYPNPNI-RGFPARRQWRP-----PGPAMGHRRNGPFYRNQQVIQRG
ENAM_platypus    TPGHGTVPDNGE---PPGLPLNASDHGGARGPKPTGPGPGQPPFRGRPANPNAPGFPAGRQRREFVHFPGRPFDRPVGRPVGRDRDHGPTYGNTPKARG
ENAM_Anoelis     PGLQ-NNMFSGNAVSTPLPTTQTSEGNGVA--QN--VLEQIRHRSKLPGNDVIQSFPSGRQHST-----MQVYNI--HKPHEHMEN---
ENAM_Xenopus     STIA-LNAQNGH---IEGPPVIAVVHNKHKNTLHG--ASRQAHYVVRAPNDPTILSN-----HQP HQKYHI---
ENAM_coelacanth  -----PNTDINPSGQGDNTSNTGNVHFGRGDNP-QNPLSNTPGQEGSPNPEFNLPGQGNMPPNTEVNPLGQGSTNPDPGYNSVGQGSIPNTRY
ENAM_bichir      -----TNPEANPPASGNGSPANGQSQPDGRDSP-NQGVNQPN-----PAQGINPMYPGNVNPFSG-----
ENAM_gar         GPNTPGSSPPKPEGAPAGPGGNTPAVGPNLPGRGDSGNAGPSSP-GVAGGFVVPGTNPLYP-NLDPFAG-----
ENAM_zebrafish   DVIAAAV-----AAV-----DPALSPE-----APAVAMEIDTTV-----V---GPDVAAGEQVL---
ENAM_trout       VDIPATV-----ATD-----IIVDPA-----LIAPI---DTIV-----AADVPAEVDAGATDV---
ENAM_fugu        D-AAAADVVDAAAADVVDAAAGSPAAAADVVDPAADAAAADVPAACI--DV--PAGAEAAAAEVPVDAADAAADVVP-----RGDATAADVPPTGV
ENAM_medaka      VDVAADV-----IAP-----EVAVDVA-----GPAAA-DI--PA-----AADVPAAGDVTATDA---
ENAM_tilapia     VDVVPVDS--APAAP-----EVAVDPA-----APATA-DI--LA-----V-----

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ENAM_human      exon9 (continued) P-RWNFFAWERKQVARPGNPVYHKAYPPTS-RGN--Y-PN-YAGNPANLRR-KPQGP-NKHPVGTTVAPLGPK-PGPVVRNEKIQNPKKPLGPKQEI-
ENAM_pig         P-RWNSFTLEGKQAVRPGYPTYYRRVYGSTA-RSN--P-PN-YAGNSANLRR-KPEGP-NKNPMVTNVAPPGP-KHGTVDQNEINIQNPKEQVQSQKERT-
ENAM_platypus    PLRWNAVTSSEKRVARPGYPMYRRFFPVAA-PGRG-F-PN-RAGNPLNLRK-NPQAP-AKHFGPEANSGPVYSQSGGLMAPREPVASPQENPPSPREKP-
ENAM_Anoelis     -----I-KQNLISRGNPSIQSEDTPTP-MGNG-WNSN-HRGNIQNTNVNPNSTN-T-----R-HIPYQQEQPHYPERNEFSQIDRV-
ENAM_Xenopus     -----VDKILVGRGGFPSPQNTIYHQEQITGKVP-L-TNIHTEKSSYIRH-VQTNSDKIPLRTVATKDGDS-F-----VREARGM
ENAM_coelacanth  NPSGQGRIPPHTRANPPSQRSMSYPTNTQRVGQKGIQPNPDYYPGSGQESIPSNTRYHTFSQGSMTPNHIYKPSGSEENIPSPPEYDPSDHGSIIPNHRYPN
ENAM_bichir      -----VFLPAGIRKVTSPSDTG-----
ENAM_gar         -----VMWPAGVRKVTSPSPSAG-----
ENAM_zebrafish   -----M-----AEAPDAALSV-----
ENAM_trout       -----GA-APLDAEANMTGGPADPTATLLA-----
ENAM_fugu        DAAAADTAHADLAAEGPAAAGSSCPDATLLA-----
ENAM_medaka      -----AENEVTGGIADPALPV-M-----
ENAM_tilapia     -----AGPLDPTAL-----

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ENAM_human      exon9 (continued) -IVPTKNPTSPWRNSQQYEVNKSNYKLPHSEGY--MPVPNFNSVDQHENSYYPRGDS--RKVPNS--DGQTQSQNLPGKIVLGSRRMPYESETNQSEL
ENAM_pig         -VVPTRDPSGPWRNSQDYGINKSNYKLQPEDN--MLVPNFNSIDQRENSYYPRGES--KRAPNS--DGQTQTQTIIPKGIVLEPRRIPYESETNQPEL
ENAM_platypus    -SFPTTRGPAVPWRKPQNYGPGKPVYRPLPSEGR--PADPGSPFPDPRENPPYPRGDATDRFLSS--GGSAPGRHFPPEGIVLKP-----EDDQPPS
ENAM_Anoelis     -PFBSSDPVGQWNKDPIYRDNDPKNSPP--EGH--PLDPQFKTM-RIDNIYNARQDA--ERFQPS--GMHQSNALSHQGVNLATRRTPFEIETHQYDW
ENAM_Xenopus     HFDPTMNTIGNDAIQIYRNNVDPNASPKVDNEHLSFLVN-KNKTPNRKIILVQHQGR--EHFPGHIMESVIESEYAPRGIFSSHRNTPSYQINKEDQ
ENAM_coelacanth  SGQVNMPNLNPNHKSFDKGGSSHIPOYSHLGHESVPPNPKYNPSGQESILPEQVYNPSGQE-RLTPYSKYNPFGQERIMPNSRYDLSGPNITQPNRYRSP
ENAM_bichir      -----STQILPGGFV-PSADGGATIP-----
ENAM_gar         -----ATQ-----GFAEPGGED---A-----
ENAM_zebrafish   -----
ENAM_trout       -----
ENAM_fugu        -----
ENAM_medaka      -----
ENAM_tilapia     -----

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C (continued)

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ENAM_human      exon9 (continued) KHSSYQPAVYPPEIIPSP-A---KEHFPAGRNTW-----DHQEISPP---FKEDPGRQEEHLPHPSHGSRGSV-FYPEY
ENAM_pig         KHSAYQPV-YTEGIPSP-A---KEHFPAGRNTW-----NQQEISPP---FKEDPGRQEEHLPHLSHGSRVHV-YYPDY
ENAM_platypus    KQKPFHPV-TSERTPR-----EPSPTKPR-----TSPEPPPP---LEEGPGRQDKSLTYPPISPRSGI-PYPDH
ENAM_Anolis      REQPFNHI-DQKR-----GQFLSAQTPTW-----NNREASQL---FQEAPPRQSSMYSSGSFNQREMP-TYSGK
ENAM_Xenopus     KLSVPNNG-DNSGN-INTAHKSEDLFRGGEFFQHNGENAAALYQKEGTASDIGTASDIGTAYDIGTAYDIGTATTGQQLESILVKHFPTNHFQVYGN
ENAM_coelacanth  SGQESISPNSRYNPSPGQEIHPNSRYNPSPGQENIPSNPNYNPSDQGRMLPHHEVNLPNKKSIQPNSGYHPSGQKNLSPRHMYNPSDHGSNPQNHEIDPL
ENAM_bichir      -----VTSSPSGNNNAVLNIPYYPSPNGG-----NINPV
ENAM_gar         -----TTPAPMGKSPALNIPFSPSEGA-----KRNPS
ENAM_zebrafish   -----
ENAM_trout       -----
ENAM_fugu        -----
ENAM_medaka      -----
ENAM_tilapia     -----
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ENAM_human      exon9 (continued) N-----PYDPRENSPYLRGNTWDERDDSPNTM--GQKESPL---YPI-NTP-DQKEIVPYNEEDPVDPTGDEVFPQNRWG-EELSFKG-----
ENAM_pig         N-----PYDPRENSPYLRNTWYERDDSPNTM--GQPENPH---YPM-NTP-DPKETIPYNEEDPIDPTGDEHFPQSRWDMEELSFKG-----
ENAM_platypus    H-----PYDPK-----GTWDEGNEPTPSS--GPAEQPGRQSPSCSPRM--QQRGEVYPYNEEDPVDPTGDEPNLGGPWE-ETANGKE-----
ENAM_Anolis      KSYNQYTHPPPP-----RAWEDREHSPTISPSPQRESSP---YPP-VSPSDQIQRTYY--RRIQPE-YEPYSRQDPWT-REQHLHD-----
ENAM_Xenopus     N-----PKNPS-----HLLSNTWQKKPDQ-----LESPP--N---NLL-GQKEIYLYPDTELYHQTNNELPLGKHWD-NEIDFSAVQFENPGS
ENAM_coelacanth  GQRHPSSPVTNTLVQEDMSHYPRSNPLRQGNSPNHETFSHGQKKYSHYDRSNHVRGKGNSPCHGSHPSGQVIHHKPQFKGNSQIH DENPWGQEGQMS
ENAM_bichir      GMRKTP-----VKNGC-----
ENAM_gar         GIRKPP-----VQVPC-----
ENAM_zebrafish   -----
ENAM_trout       -----
ENAM_fugu        -----
ENAM_medaka      -----
ENAM_tilapia     -----
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ENAM_human      exon9 (continued) -----GPTVR--HYEGEQYT---SNQPKKEYLPYSLDNPSKPREDFYSEFYWPSPDENFPSYNTASTMPPPIESRGYYVNAAGPEESTLF
ENAM_pig         -----DPTVR--HYEGEQYT---SNQPKKEYLPYSLDNPSKPREDFLYGEFYWNPEENFPSYNTAPTSSPVESRGYYANNAVQEEESTMF
ENAM_platypus    -----DPS-R--YHKSPQYPQSRATEPDYPPYEGGLEK-HRDFPYGDFYPRGPEDNLPYNGADQ--LPVEGRGYTHDAVGQGGPLQF
ENAM_Anolis      -----PD-N--QYSNSPYN---PTHHTYQKYTTENPPSEISNLPYRKINQWTQEEHSPVH-TAGHL-RQMENVPYRTNSKFEQGE-RKF
ENAM_Xenopus     STYYMKDSQRQKQWPTASNNRVELDRQ--TDQPKYLLISKLD-----PAKNTDVS--TFTNEPMAQNGYITNQEAY---LQS
ENAM_coelacanth  NAGRIPSNRGDSSPYGLFLSENRESSPYLASKPVNQGIISPNHEVYSSAQGGNRPYTGMIPTDKIIISPNHGPSSSIQGDLSQNYRGYQSGKERNQPYT
ENAM_bichir      -----NMG-----NHGPTTV-----
ENAM_gar         -----NKGPP-----HGPY-----
ENAM_zebrafish   -----
ENAM_trout       -----
ENAM_fugu        -----
ENAM_medaka      -----
ENAM_tilapia     -----
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ENAM_human      exon9 (continued) PSRNSWDHRIQAQGRERRPYFNRIWDQATHLQKAPARPDPQKGNQPPYYSNTAGLQKNPIWHEG-E-NLNYGMQITRMNSPEREH-----SSFPNF
ENAM_pig         PSWSSWDPRQAQGGKEGRPYLNRNFWDQSTNLYKTP TSSPHQKENQPYSNNSPAGLQKNPTWHEG-E-NLNYGMQITRLNSPERDH-----LAFPD
ENAM_platypus    PSRDSWDWGLRSAAQERRAPYDERSDWHAINS---PKSTAGATETLSPSHSVPA GIRGKPHEESV-D-WSYRGGQVSDLGVP AEAG-----PALMEP
ENAM_Anolis      QNVGSGSPQQRNTFQEE-AQYAERTWVPQRV-----DPSAQKETAPYYNIYSTDFRGNPTHVEDRERI IHMNAPISTENAPRRRH-----YLD RMG
ENAM_Xenopus     PNN--KSPFIFNKPSTDGILYIPIDELHDS-----QNNPVHLKLT-----YSKAHSENHSPGVFRSLQEV
ENAM_coelacanth  EVIPRGQKSIYSAPSGQRGNSENLRINPSGNSENLFYPERKPSGKESDLPSYRVYVKQGGENSPYSQPV-PSSQRIMTSDPLLQSTQKGTMPYFTLNS
ENAM_bichir      -----EPVAPSAGNSEIPSFG-----GILMA-----
ENAM_gar         -----GNGE-----QPGFVDPMPNPDAEGIL A-----
ENAM_zebrafish   -----
ENAM_trout       -----
ENAM_fugu        -----
ENAM_medaka      -----
ENAM_tilapia     -----
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C (continued)

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ENAM_human      exon9 (continued)
ENAM_pig          IPPSYPSGQKEAHL-----FHLS-QRG-SCCAGSSTGPKDNPLAL-----
ENAM_platypus     IPPDYPGGQKESHV-----FHLS-QRG-PCCAGGSMWPKNNPLAL-----
ENAM_Anolis       VARSDPADPREASS-----YPVAVARS-SCCAGGSPGPRDVPLAPDPYPLALPGYLPDPLDYPPLDLDRPDYLPAPRDPYPPP
ENAM_Xenopus      YSDDYPKERRMIT-----SQST-ANQ-LCCADDSFMPMPRENRLAP-----
ENAM_coelacanth   NPNIYPSSLRRQSLNPRKTGHYSCITPLTGVDPTLEHSN-ALIILVCCKLNDM-GQSHPSAN-----
ENAM_bichir       PGQKTNSPYISENPSDQRGTSFKNRIYPAGQARLPYPASSVFSQRDNHPIHGTFNPSCQEDIPPNQDTNLLSKIGFTPTYLENPSEQRQSGFCPRNNQL
ENAM_gar          -----AAEAGSDGEGRT-----
ENAM_zebrafish    -----AADA-----
ENAM_trout        -----
ENAM_fugu         -----
ENAM_medaka       -----
ENAM_tilapia      -----

ENAM_human      exon9 (continued)
ENAM_pig          -----QDYTPSYGLAPGENQDTSPLYTDGSHTKQTRDII-SPTSILPGQRNSSE----KRESQNPFRDGVSTLRRNTPCSI
ENAM_platypus     -----QDYTQSFGLAPGENPDTSIGYAEDSHIKYARQTV-SPTSIVPGQRNSSEKILPGESQNPSPFKDDVSTLRRSTPCSV
ENAM_Anolis       DTPNYSPLDTPDYPPHYPLAPQDYSSPLGLSLWELQVGGPRSPGGSRSRHVQQAA-SPVGYPAIRRNGETEAPTRREEPAPSRDGLA-PGRSPPCSL
ENAM_Xenopus      -----LRSAPQFRHALWEAKESST-YPDGSHANYVRHAH-SPAGIQA--NNPLKNGGREQEELGAFFVENAGSEKSLPSCSN
ENAM_coelacanth   -----EDNVHHLKVVLNEYKESVP-HQDQTHRQHTRDVIESPKFSI--Q-NNVMTVRPNSSNNHDNIQSSRGKSSDSVPCTS
ENAM_bichir       CQRSNSPDFKGNSPITERKSLGWRERTHSVGATPIHGNDHPDSFSPRKSQFPADNFPYQAGNPETPRNQFVDRENMPITRDSPLGSEGNMCKPSLSEP
ENAM_gar          -----IHGDS-----
ENAM_zebrafish    -----
ENAM_trout        -----
ENAM_fugu         -----
ENAM_medaka       -----
ENAM_tilapia      -----

ENAM_human      exon9 (continued)
ENAM_pig          KNQLGQKEI-MPFPEASSLQSKNTPCLKNDLGGDGNNILEQVFEFN-QLNERTVDLTPEQLVIGTPDEGSNPEGIQSQVQENESERQQQRPSN--ILHL
ENAM_platypus     KSQLSQRGI-MPLPEANSLQSKNTPCLTSDLGGDGNNVLEQIFEGN-QLNERTVDLTPEQLVFGTPDKEPRPEGIPNEMQGNESERQQQRQSS--ILQL
ENAM_Anolis       RPRASPRAG-LAFPEAGLPPAKPAPCFKSRRLRGDVDSVPEQILDPD-PPVAGNDGPAPGDLAVVPERDPGFPVGIKRGDP---EPGPEAGAPG--FQWV
ENAM_Xenopus      SH-LSQDSKQEANLQRLGFKFRNMPCHGSNIRGDRHNPLAHLVGTGQSFGRGTLNFLPEQ-----FPQPHGIQSEALVSEDDGKEYAALG--AKRI
ENAM_coelacanth   KPSYEQNSE-YPY-----AFPKKQCS--YRGDADSYLPPSQDESISLKK--RFLASENVYML-ANTPEPDL-PDRRI--KDDGKVKKGIGKLEIL
ENAM_bichir       -----RVNLFDRDNPALFSTTTNANNFEEGISTTSPKKEMPPPGVDLTRHATKSWIPAAAGDMLPAPIKKA
ENAM_gar          -----RV
ENAM_zebrafish    -----
ENAM_trout        -----
ENAM_fugu         -----
ENAM_medaka       -----
ENAM_tilapia      -----

ENAM_human      exon9 (continued)
ENAM_pig          PCFGSKLAKHHSST-----GTPS-----SDGRQSP-----FDGDSI--TPTE-----NPNTLVELA--TEEQFKS-----INVDP-
ENAM_platypus     PCFGSKLANHYHTSSI-----GTPS-----SLGRQDS-----FDGDPI--MPTE-----TPNSLAGLA--TGAQFQN-----INVDP-
ENAM_Anolis       PCFGSETRDSK-SLL-----GMDGRSPWQREAGPIRAGVHRTTRDPGRGLWASDRGAFRGPQP--SPFIRTF TALQDPPVGSGR--SGTTGSR-----LLVAP-
ENAM_Xenopus      PCFGSWLKQYL-SNT-----EVPS-----DDQQRDP-----FYGENP--VPTE-----RPNSIPKPEPISSSFLS-----NGVEE-
ENAM_coelacanth   TCPQDQEIHTNKKAINIAKTRPTA-----SLLNHDK-----LSADAIP-----VLNTEQ
ENAM_bichir       PCFGDRTSPTS RDKIMSFGGSATLAGVRKAPPGNGDSMVLAVKPNLVPPASKTTVATDPKNDMALSTLNKFGTEKDALSAPPIAVNEDPRENRAPDCVLLRD-
ENAM_gar          PCRVHDTATAGKESNFYY-----PCVVHDSPAQAEENHYYY-----
ENAM_zebrafish    -----
ENAM_trout        -----
ENAM_fugu         -----
ENAM_medaka       -----
ENAM_tilapia      -----
```

Figure S1. Multiply aligned amino acid sequences encoded by matrix SCPP genes, Related to Figure 1.

(A) Multiply aligned amino acid sequences encoded by *AMEL* and *SCPP5*, their chromosomal locations, and expression of bichir *scpp5* in the jaw and skin. Aromatic residues, negatively charged residues, and positively charged residues are shown in sky blue, blue, and brown, respectively, while Pro, Gln, and potentially phosphorylated Ser residues (potential pSer residues: Ser residues in Ser-Xaa-Glu or Ser-Xaa-pSer sequences, where Xaa represents any amino acids, are known to be phosphorylated in SCPPs.) (Kawasaki and Amemiya, 2014) are highlighted in yellow, blue, and green, respectively. Uninterrupted Pro-Xaa-Yaa (Yaa also represents any amino acid; PXY) repeats are shown with dashed underlines. The three conserved modules, the aromatic residue-rich region (YY), the Pro/Gln-rich region containing uninterrupted PXY repeats (P/Q), and the hydrophilic C-terminus (DE) are shown at the bottom. Unaligned sequence gaps are shown by dashes. Amino acid sequences were retrieved from GenBank (accession numbers for *AMEL*: NP_873632.1 for humans, NP_998965.1 for pig, reconstructed from GCA_000002275 for platypus, XP_003228746.1 for *Anolis*, AAC78133.1 for caiman, AAZ23149.1 for axolotl, NP_001107153.1 for *Xenopus*, AYU58914.1 for lungfish, and reconstructed from GCA_000225785.1 for coelacanth; accession numbers for *SCPP5*: AYU58917.1 for bichir, XP_015200896.1 for gar, XP_035273951.1 for eel, NP_001138708.1 for zebrafish, reconstructed from GCA_000180675.1 for stickleback, NP_001032946.1 for fugu, reconstructed from GCA_000188235.2 for tilapia, and reconstructed from GCA_002234675.1 for medaka). Note that all teleost species used for multiple amino acid sequence alignments (A, B, and C) belong to different orders. In sarcopterygians, *AMEL* is located within intron 1 of *ARHGAP6*, while in actinopterygians *SCPP5* is located within a P/Q-rich SCPP gene cluster (Kawasaki et al., 2017). In *L. chalumnae*, locations of genes are shown as contig names (JHxxxxxx). Expression of bichir *scpp5* was detected in the jaw and skin that secretes the ganoin matrix on scales, but not in the skeletal muscle (negative control). Bichir *gapdh* (glyceraldehyde 3-phosphate dehydrogenase) was used as the positive control.

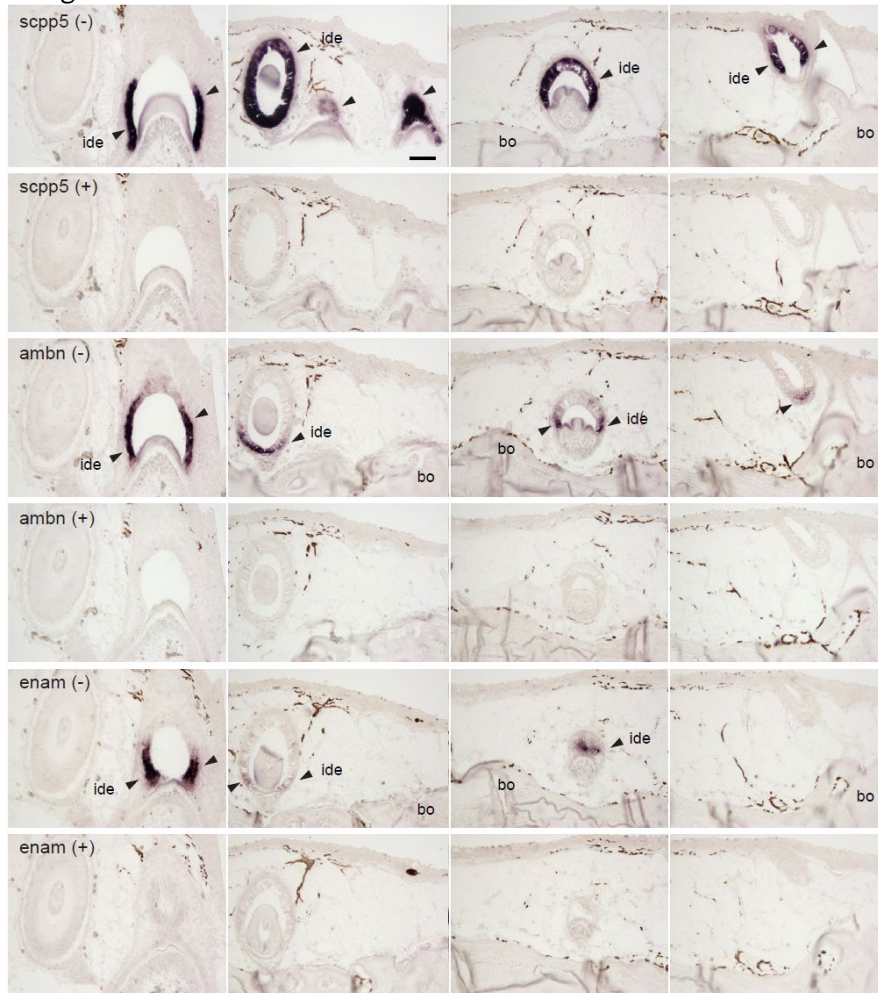
(B) Multiply aligned amino acid sequences encoded by *AMBN*. Cys residues is shown in red. See the legend above. *AMBN* in sturgeon, medaka, and tilapia has a small last exon, and the C-terminus is shown by a period. The amino acid sequences shown under exon 6 of human *AMBN* are encoded by two different exons in bichir, sturgeon, and gar (separated by an underscore). In a previous study, *ambn* was named *scpp6* in teleosts (Kawasaki, 2009). However, conservation of the amino acid sequences encoded by exons 11 and 12 (corresponds to exon 12 of human *AMBN*) between *AMBN* and *SCPP6*, the absence of the entirely untranslated last exon in both *AMBN* and *SCPP6*, the presence and locations of four potential pSer residues, and an equivalent chromosomal location of gar *ambn* and zebrafish *scpp6* (between *scpp3* genes and *scpp7*) implies the orthology of *AMBN* and *SCPP6* (Qu et al., 2015; Braasch et al., 2016; Kawasaki et al., 2017). In the present study, the orthology of *AMBN* and *SCPP6* was confirmed by a similar expression pattern during tooth development. Amino acid sequences were retrieved from GenBank (AAG35772.1 for humans, XP_007667389.2 for platypus,

AAK92227.1 for caiman, XP_016846623.1 for *Anolis*, XP_002938667.2 for *Xenopus*, AYU58913.1 for lungfish, XP_006011890.1 for coelacanth, AYU58915.1 for bichir, reconstructed from SRX424534 for sturgeon, AMD08894.1 for gar, NP_001138709.1 for zebrafish, reconstructed from GCA_002234675.1 for medaka, reconstructed from GCA_000188235.2 for tilapia, and NP_001032945.1 for fugu).

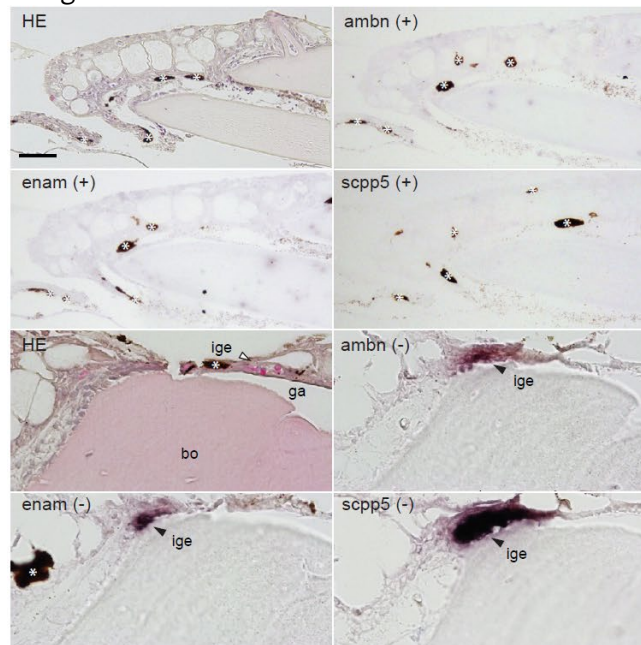
(C) Multiply aligned amino acid sequences encoded by exons 3, 4, 5, 6, 7, 8, and a 5' half of exon 9 of *ENAM*. The amino acid sequence of porcine 32-kDa enamel (Tanabe et al., 1990; Yamakoshi et al., 1998) was underlined. Small amino acids (Ala and Gly) in the 32-kDa enamel and corresponding regions are highlighted in grey. In the region corresponding to exon 6 of human *ENAM*, many small duplicate exons were identified in gar and bichir *enam* genes but are not shown in the figure. Amino acid sequence similarities in the portion located C-terminal to the 32-kDa enamel (not highlighted or not shown in color) are low or undetectable between phylogenetically distant vertebrates (e.g., tetrapods and teleosts). In teleosts, *enam* was originally referred to as *fa93e10* (Goldsmith et al., 2003). However, sequence similarities in the 32-kDa enamel and its N-terminal region encoded by *ENAM* and *FA93E10*, the absence of the entirely untranslated last exon in both *ENAM* and *FA93E10*, the presence and locations of four pSer residues, and an equivalent chromosomal location of far *enam* and zebrafish *enam* (immediately downstream of *scpp5*) imply the orthology of *ENAM* and *FA93E10* (Qu et al., 2015; Braasch et al., 2016; Kawasaki et al., 2017). In the present study, the orthology of *ENAM* and *FA93E10* was confirmed by a similar expression pattern during tooth development. Amino acid sequences were retrieved from GenBank (AAG43242.1 for humans, NP_999406.1 for pig, reconstructed from GCA_000002275.2 for platypus, ADJ67842.1 for *Anolis*, NP_001139215.1 for *Xenopus*, reconstructed from GCA_000225785.1 for coelacanth, AYU58916.1 for bichir, AMD08897.1 for gar, NP_001139028.1 for zebrafish, CDQ64219.1 for trout, reconstructed from GCA_000180735.1 for fugu, reconstructed from GCA_002234675.1 for medaka, and XP_003454248.1 for tilapia).

A

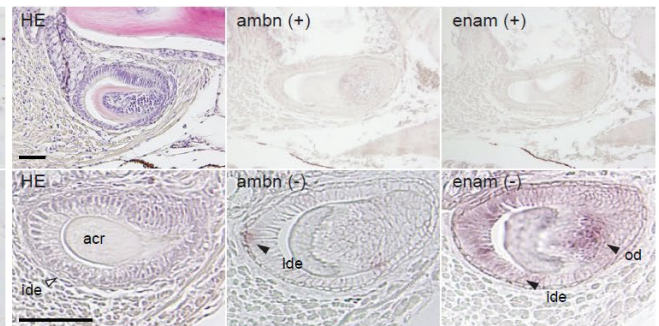
ISH: gar teeth



ISH: gar scales

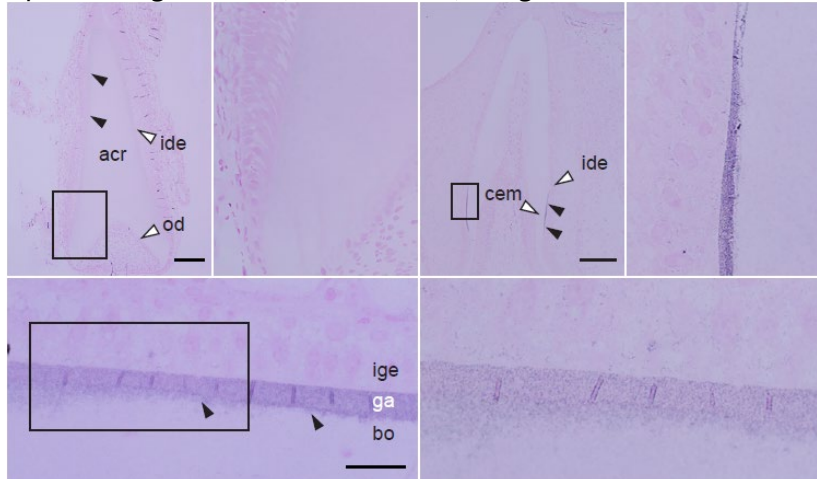


ISH: zebrafish teeth



B

Optical IHC: gar acroдин, collar enamel, and ganoin

**C**

TEM IHC: gar acroдин, collar enamel, and ganoin

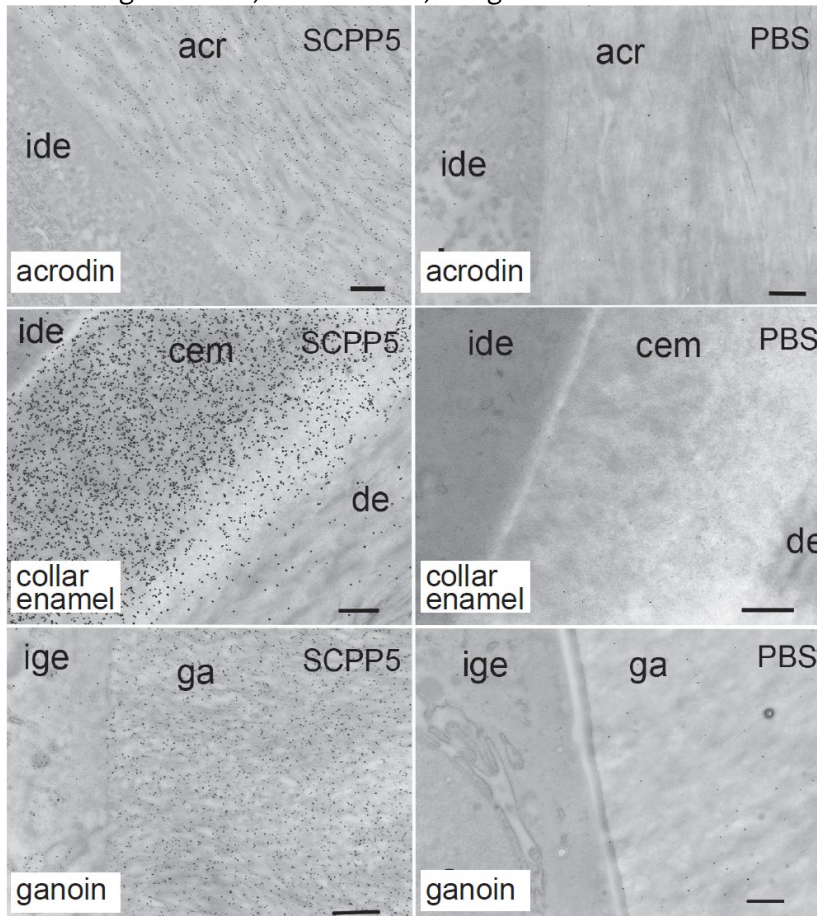


Figure S2. Expression of gar and zebrafish *scpp5*, *ambn*, and *enam* genes in teeth and/or scales, and distribution of gar Scpp5 in teeth and scales, Related to Figures 2-4.

(A) ISH analysis of gar teeth, gar scales, and zebrafish teeth. Expression of *scpp5*, *ambn*, and *enam* genes were detected using antisense (-) probes, but not using sense (+) probes, which serve as negative controls. Images of HE staining are shown for gar scales and zebrafish teeth. All our ISH results are summarized in Figure 2P.

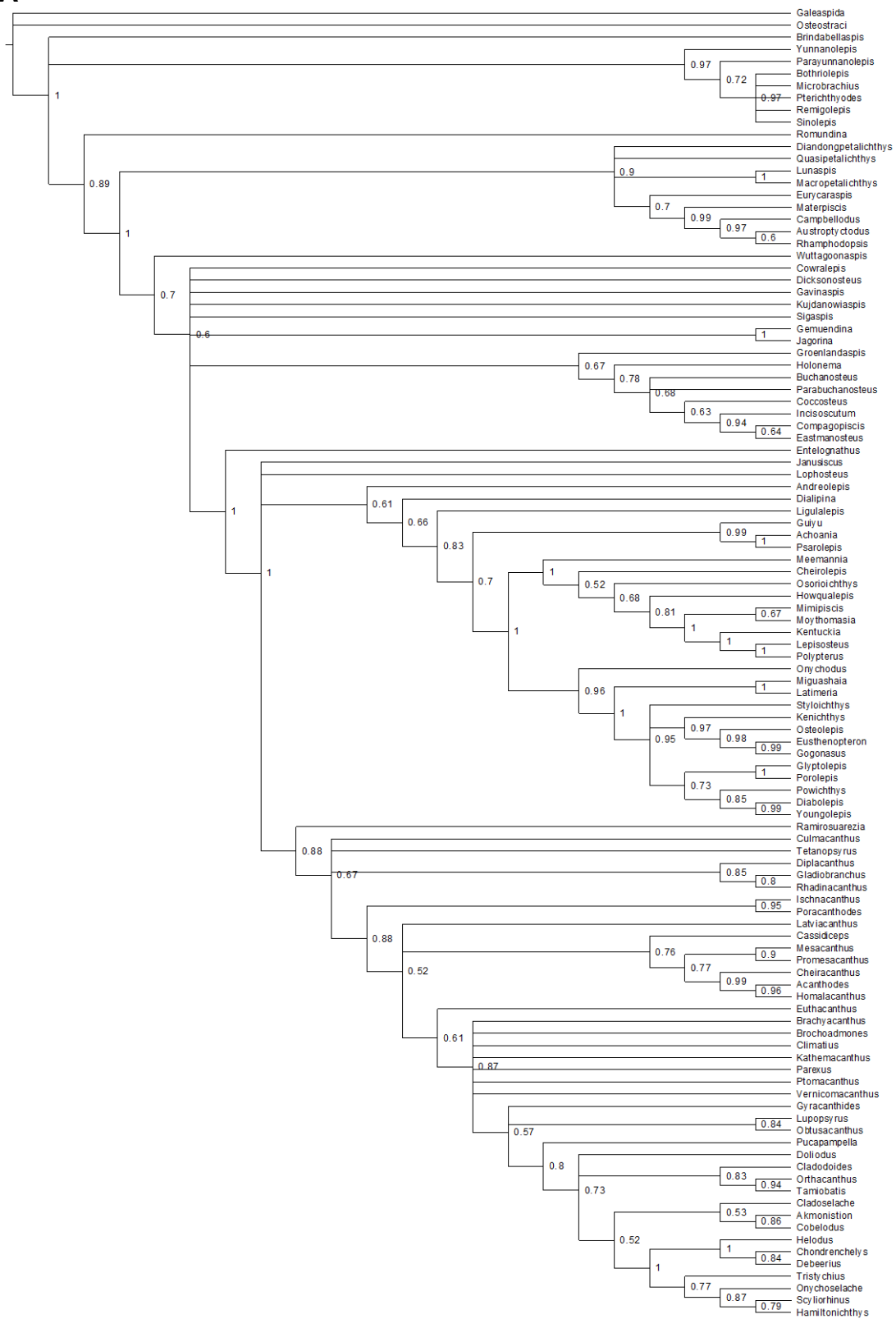
Abbreviations: acr, acrodin; bo, bone; ga, ganoin; ide, inner dental epithelial cells; ige, inner ganoin epithelial cells; od, odontoblasts. Scale bar, 50 μ m.

(B) Original images for optical IHC analysis (Figures 3A-3F) without enhancing image contrast. See the legend of Figure 3. Scale bar, 100 μ m (upper row) or 20 μ m (lower row).

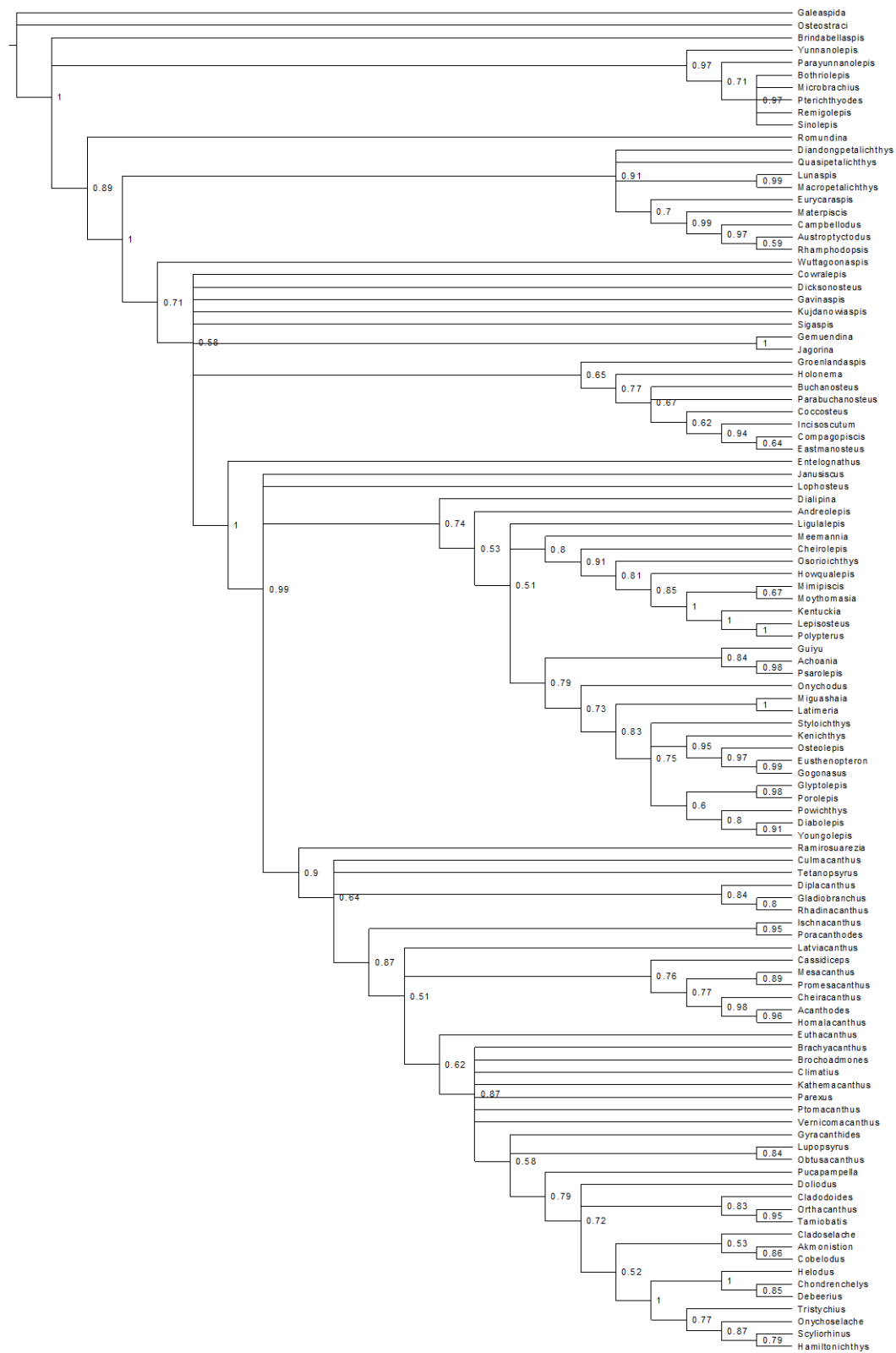
(C) TEM IHC analysis of gar *Scpp5* in acrodin, collar enamel, and ganoin. PBS was used as negative controls.

Abbreviations: acr, acrodin, cem, collar enamel; de, dentine; ga, ganoin; ide, inner dental epithelial cells; ige, inner ganoin epithelial cells. Scale bar, 500 nm.

A

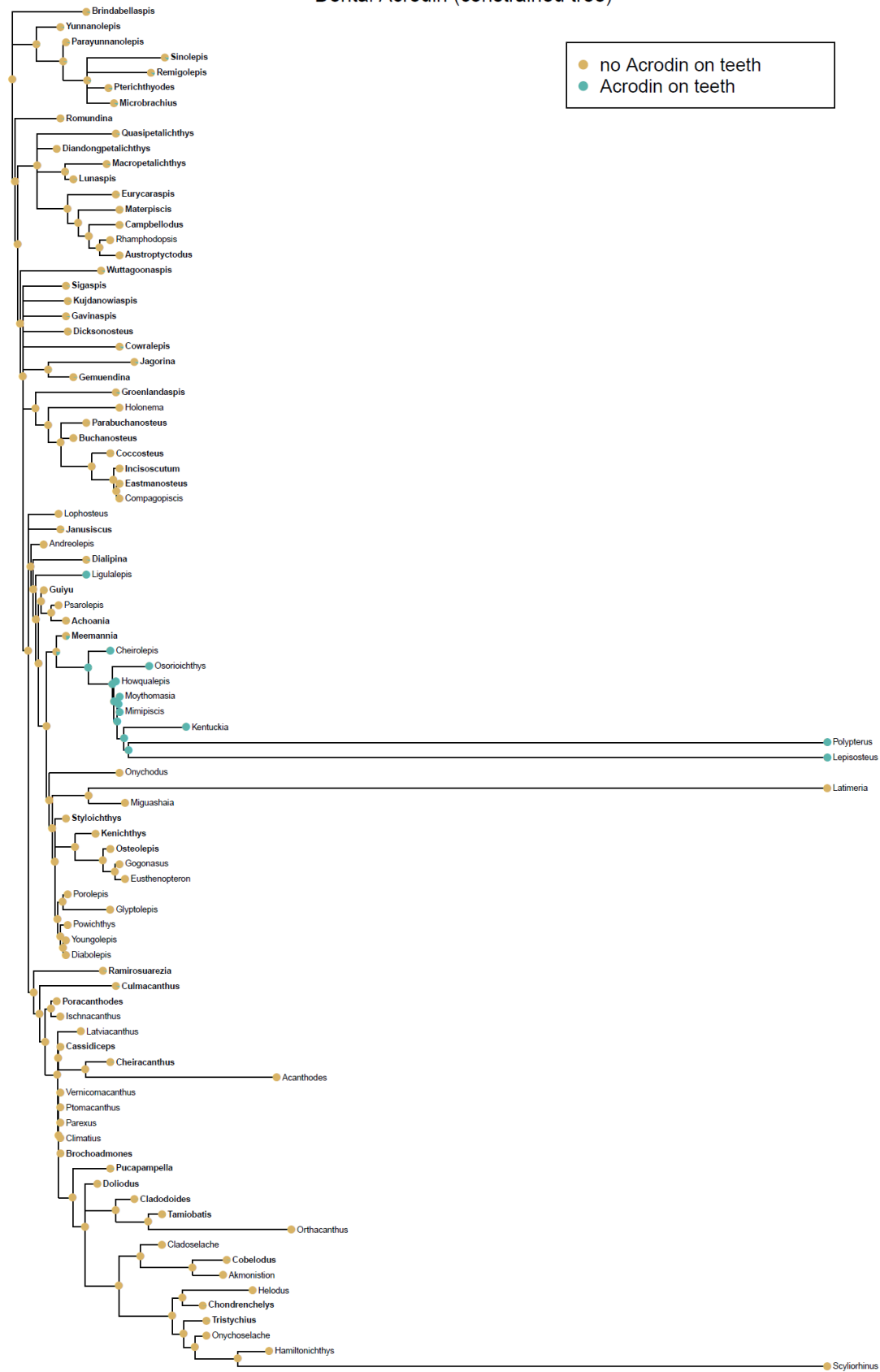


B



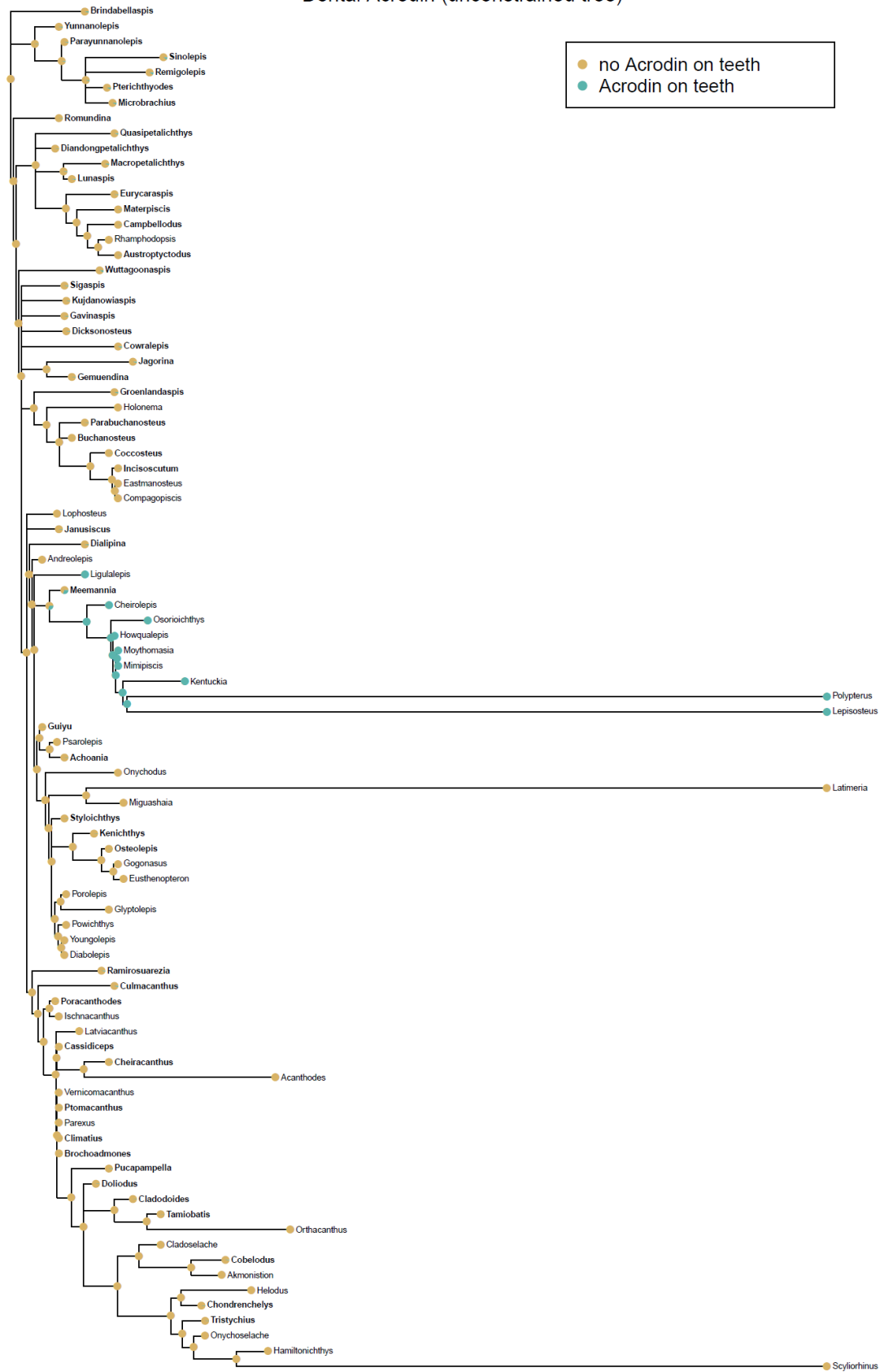
C

Dental Acrodin (constrained tree)



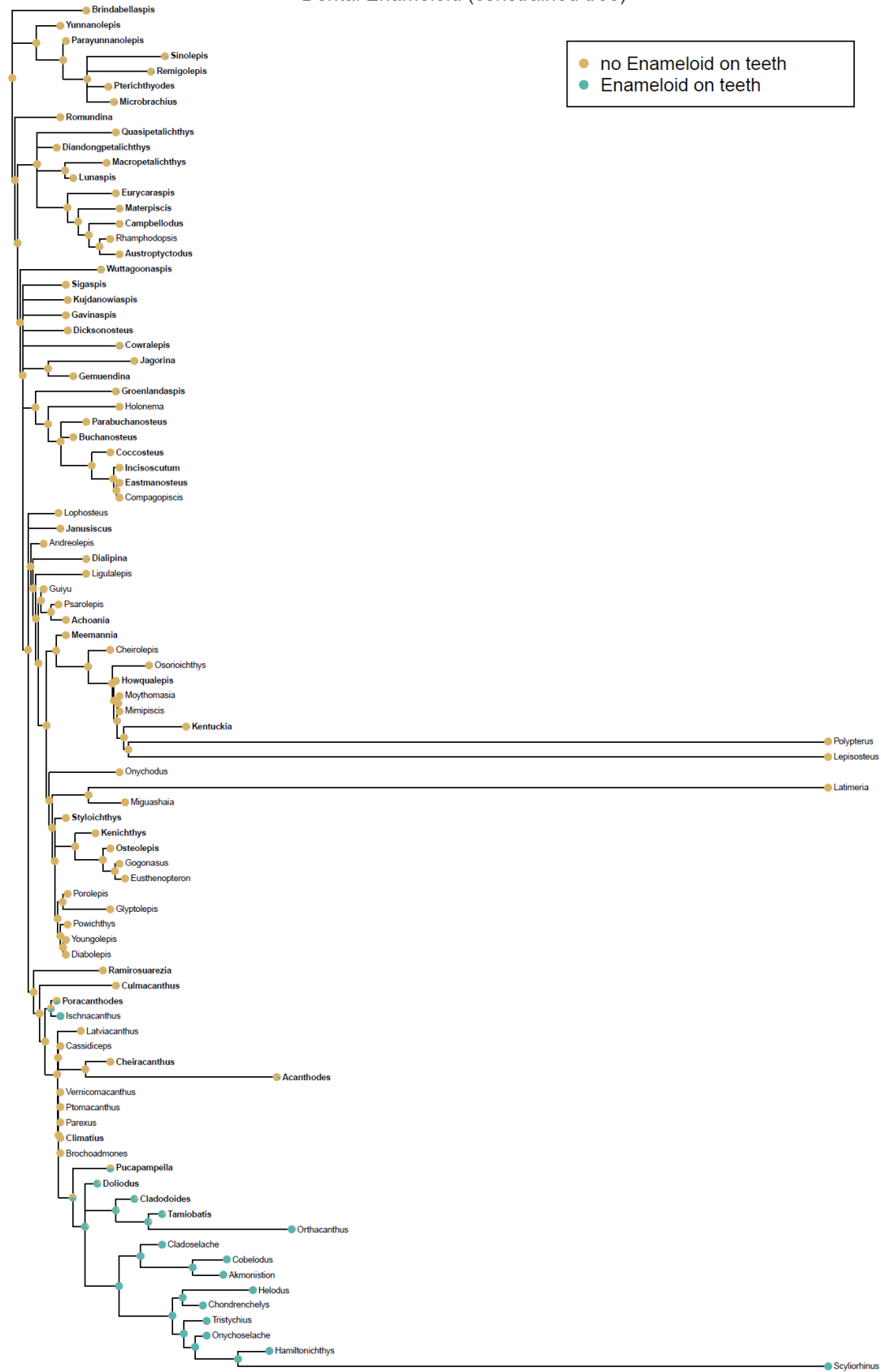
D

Dental Acrodin (unconstrained tree)



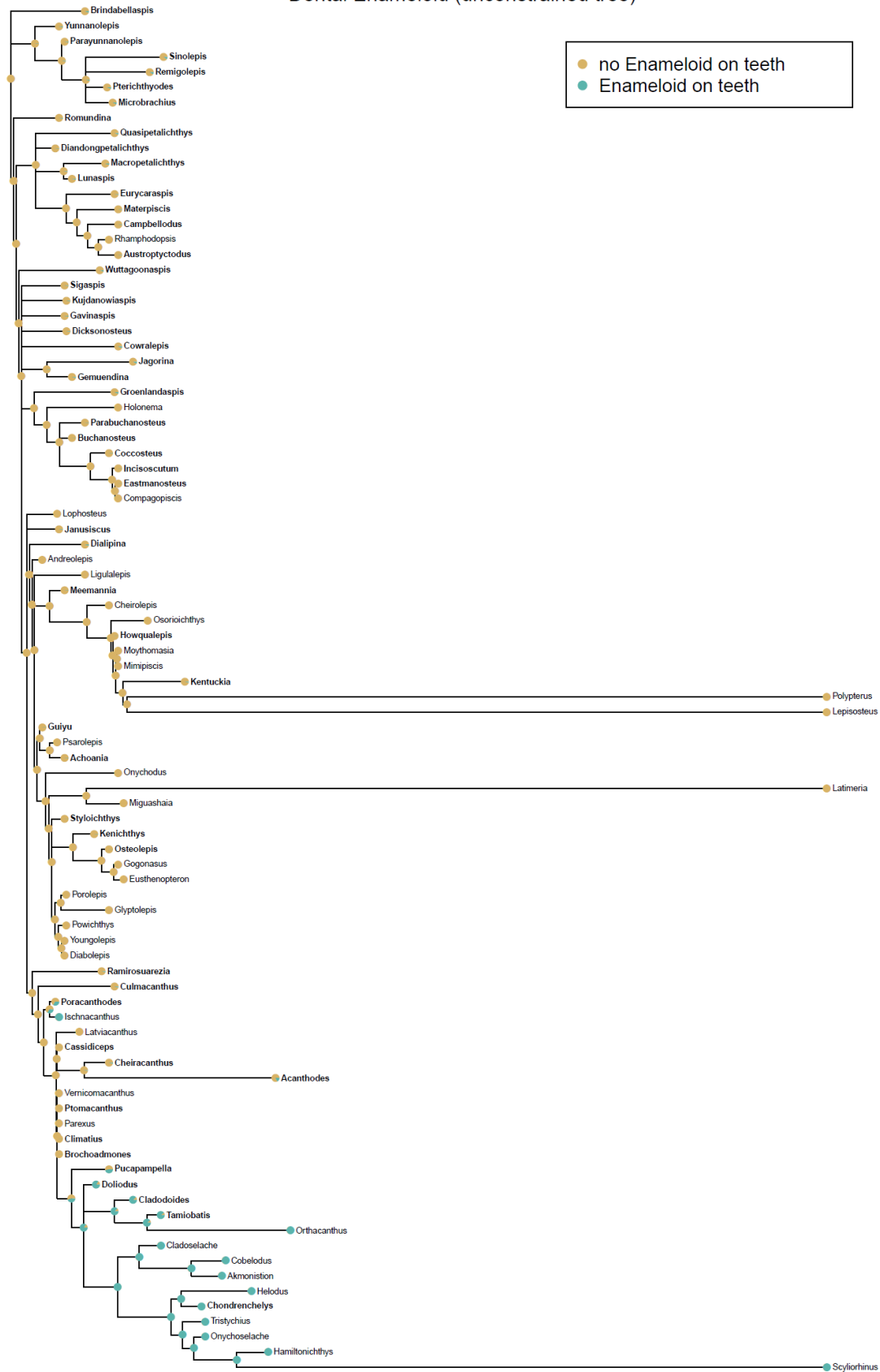
E

Dental Enameloid (constrained tree)



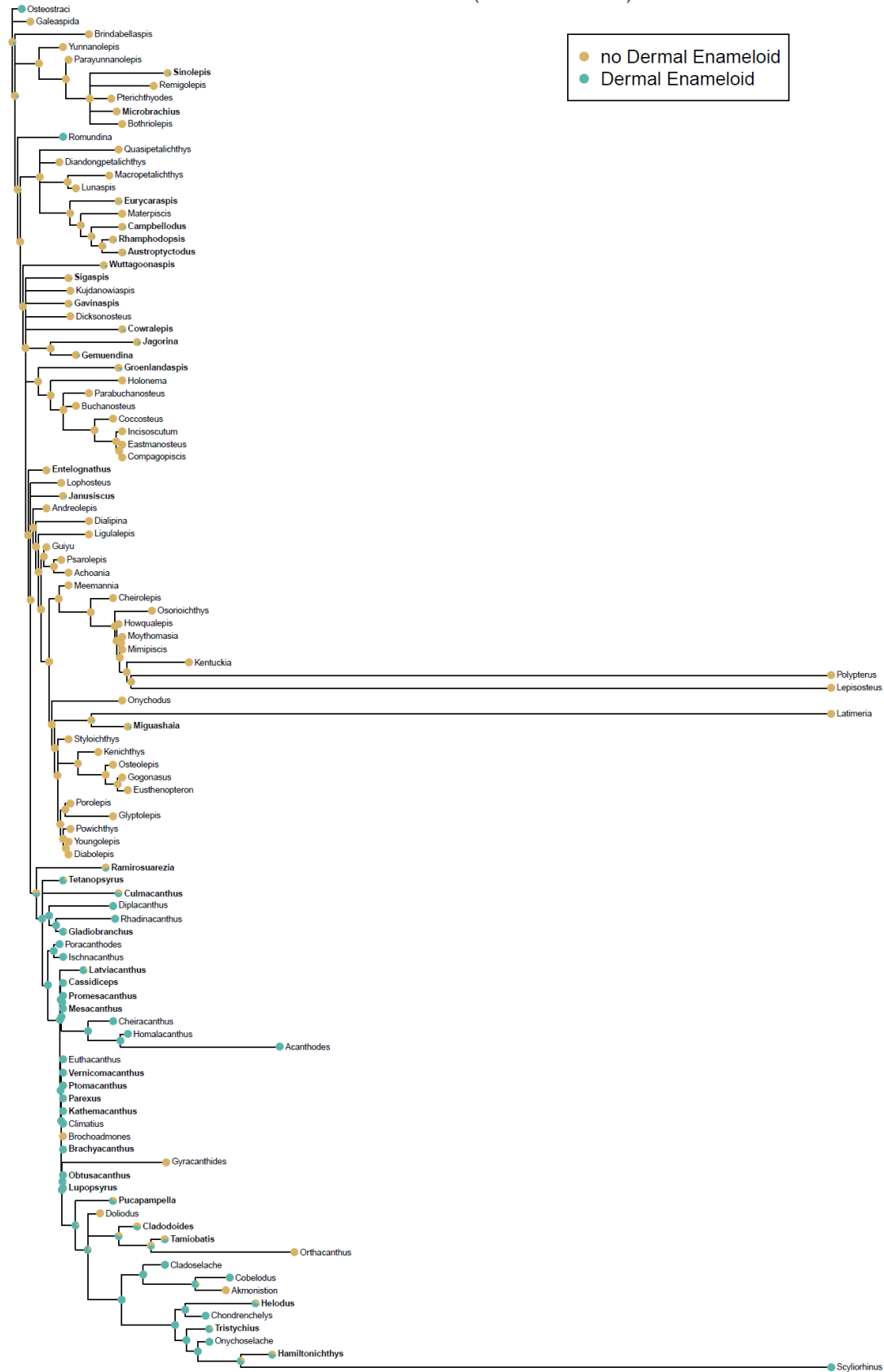
F

Dental Enameloid (unconstrained tree)



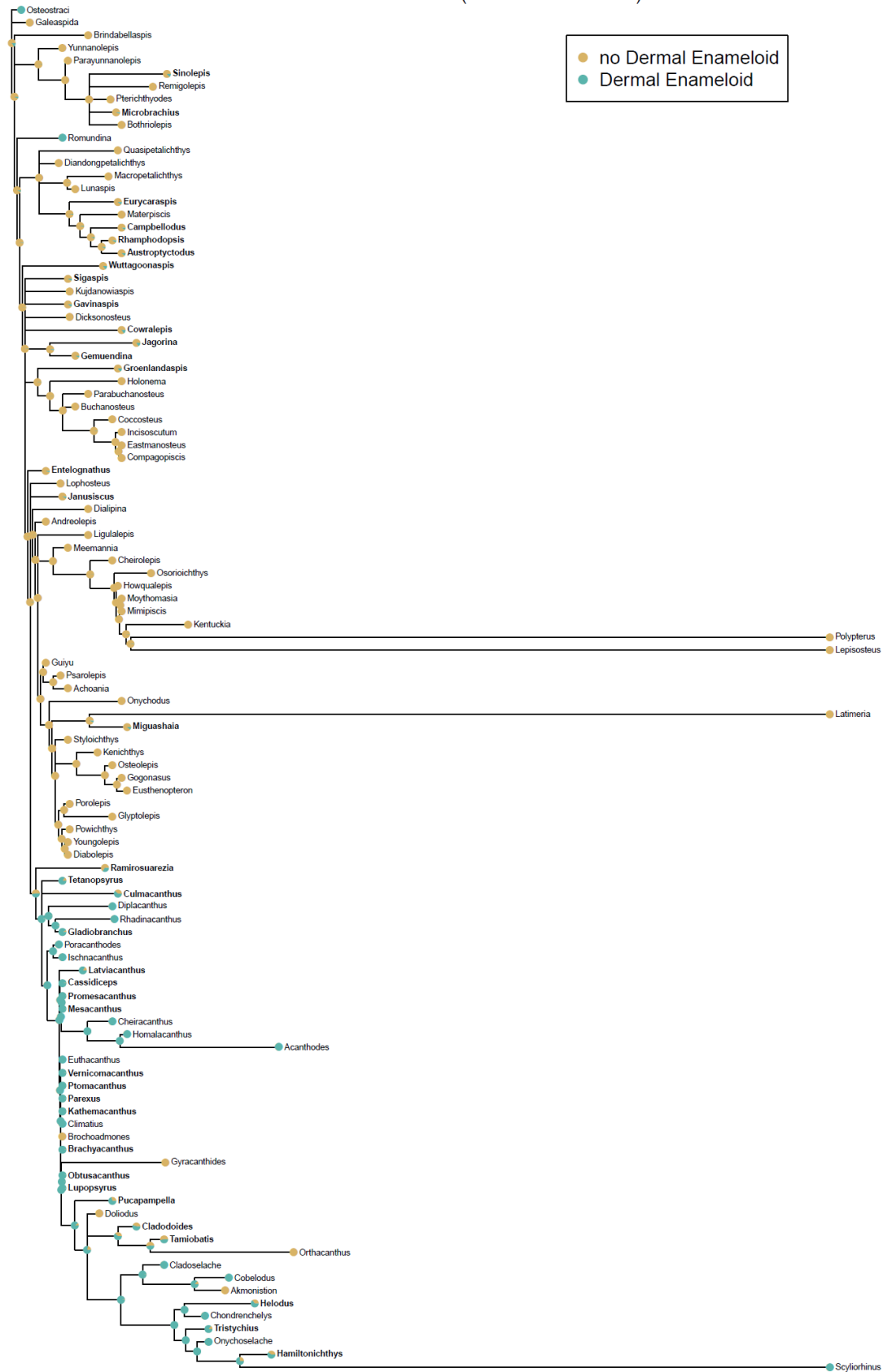
G

Dermal Enameloid (constrained tree)



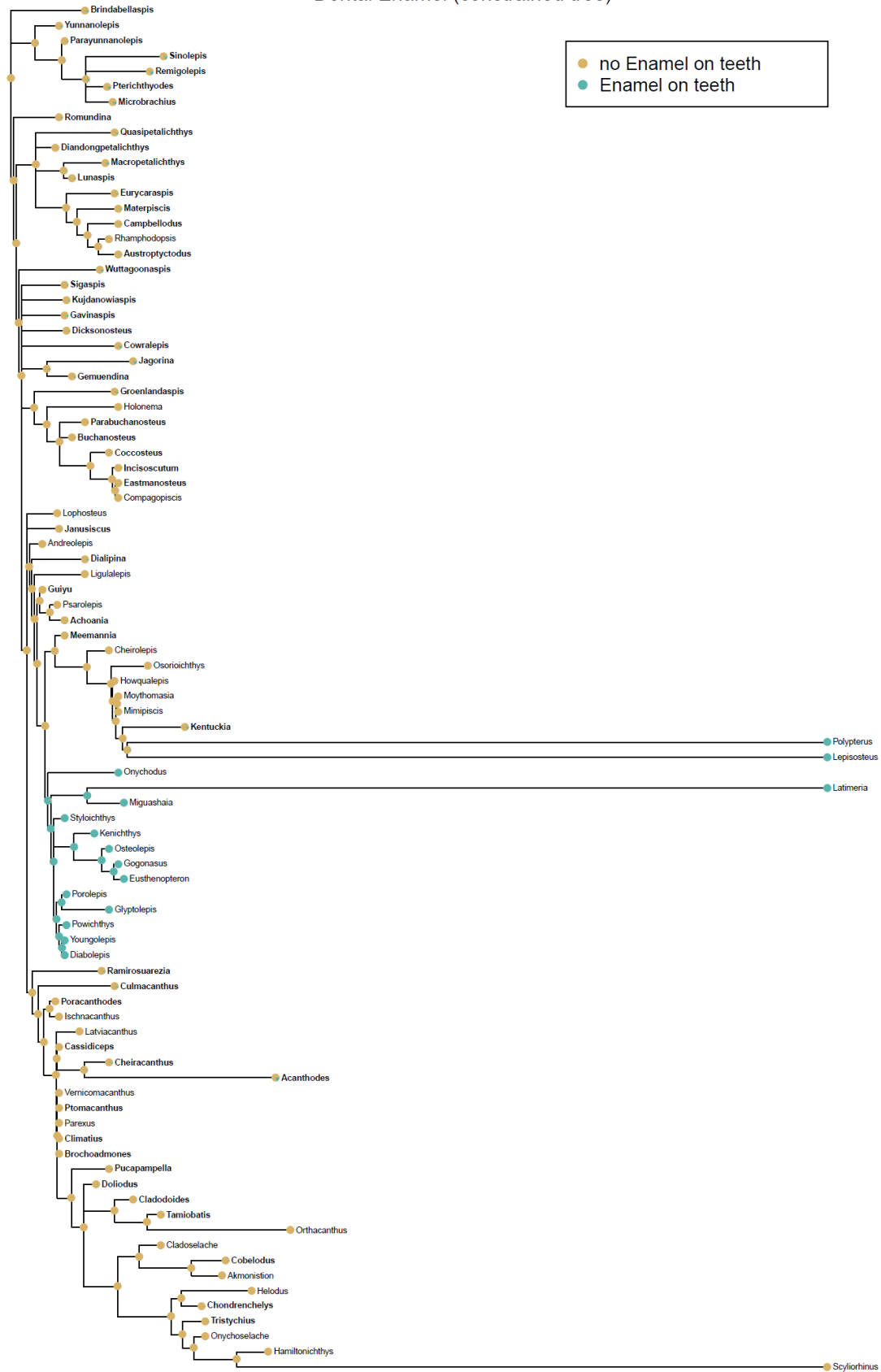
H

Dermal Enameloid (unconstrained tree)



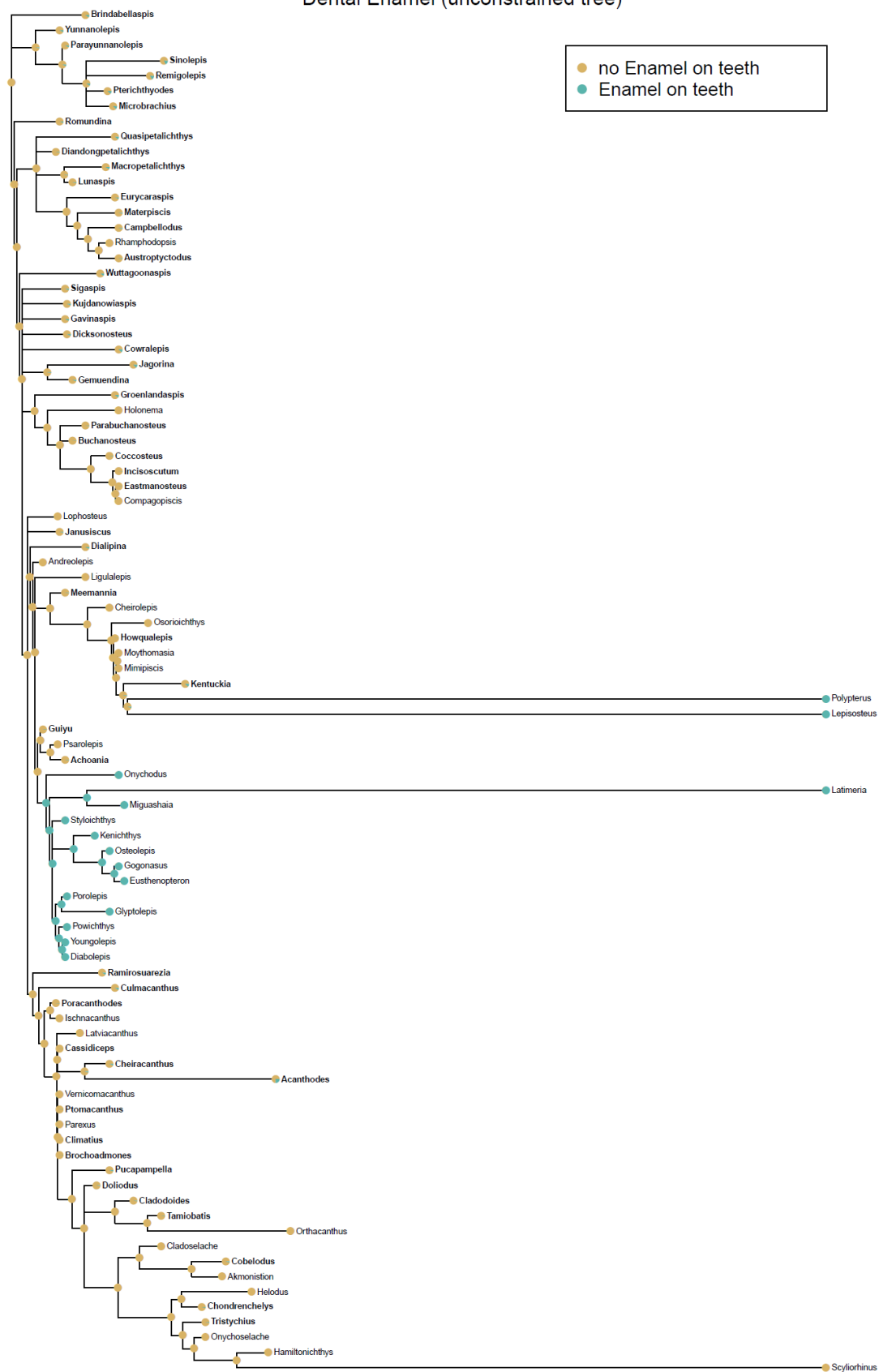
I

Dental Enamel (constrained tree)



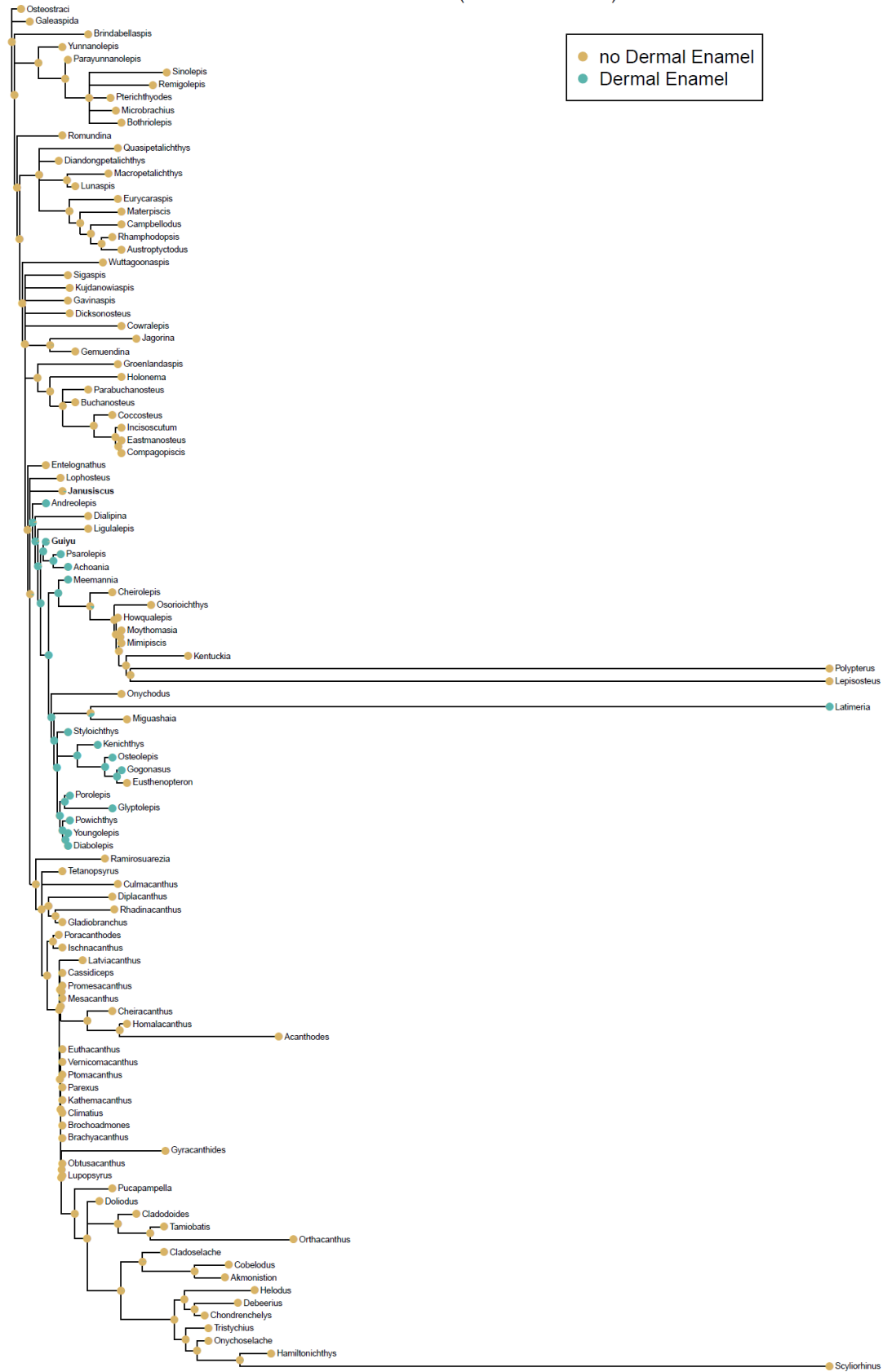
J

Dental Enamel (unconstrained tree)



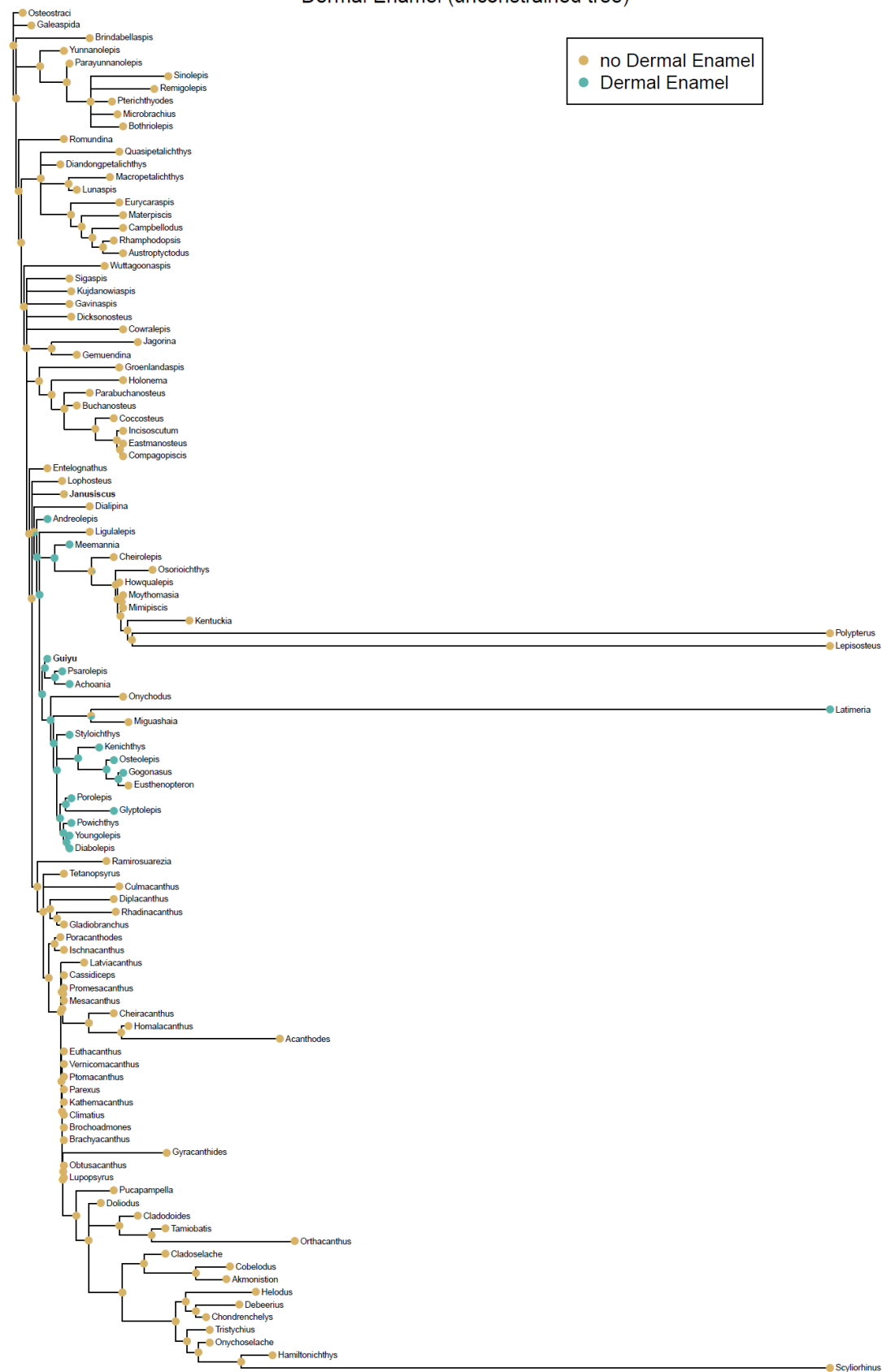
K

Dermal Enamel (constrained tree)



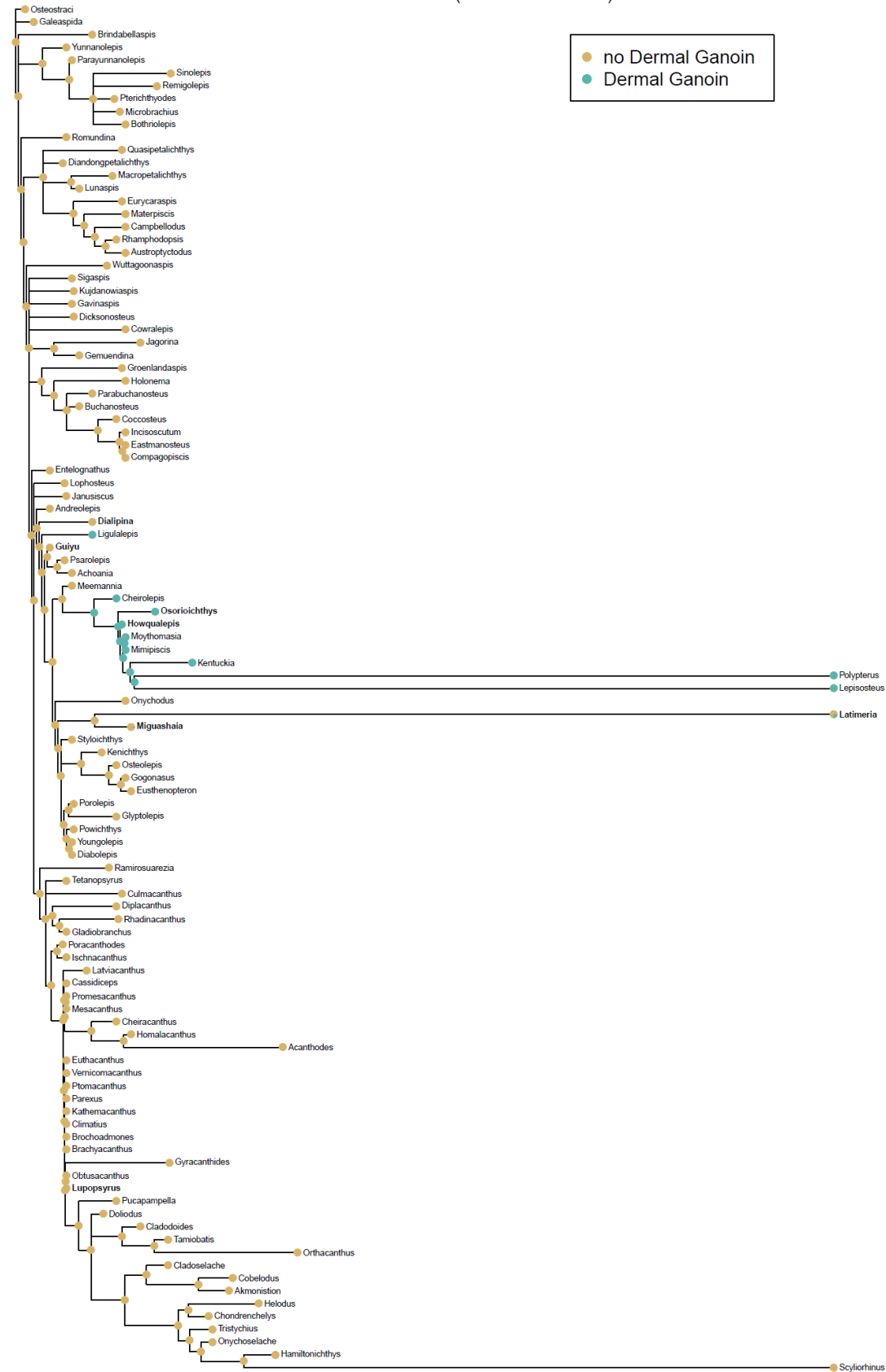
L

Dermal Enamel (unconstrained tree)



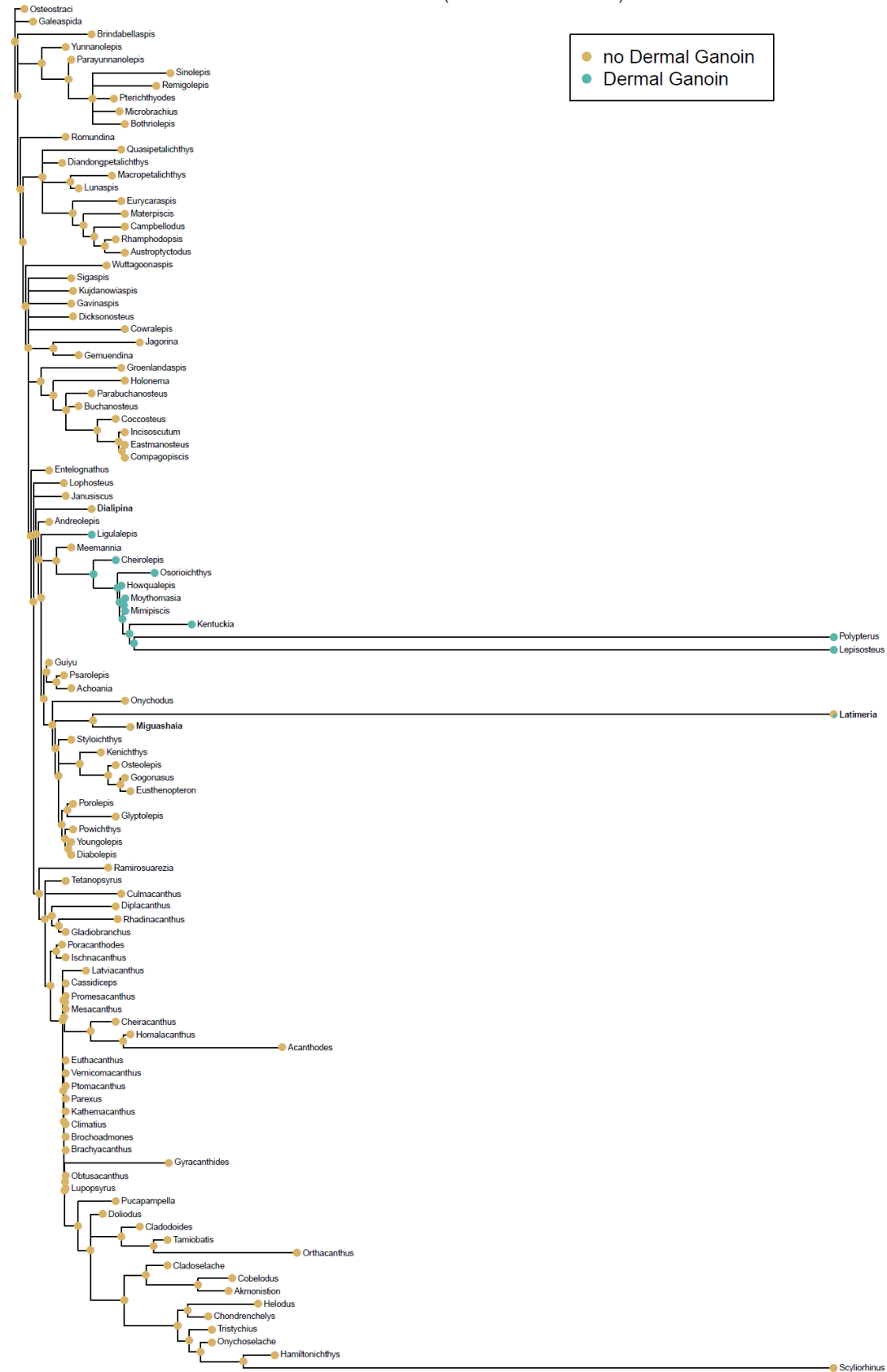
M

Dermal Ganoin (constrained tree)



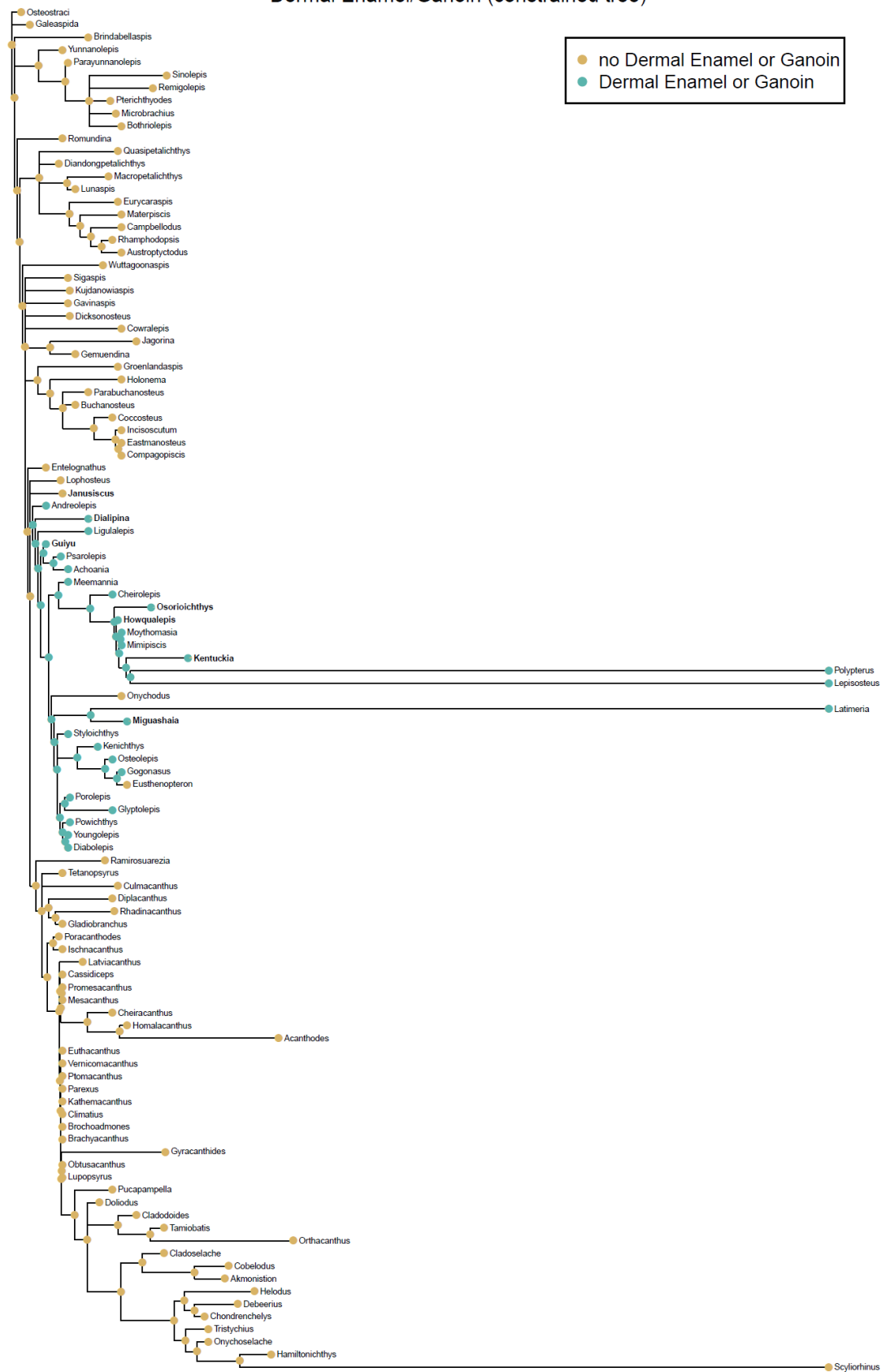
N

Dermal Ganoin (unconstrained tree)



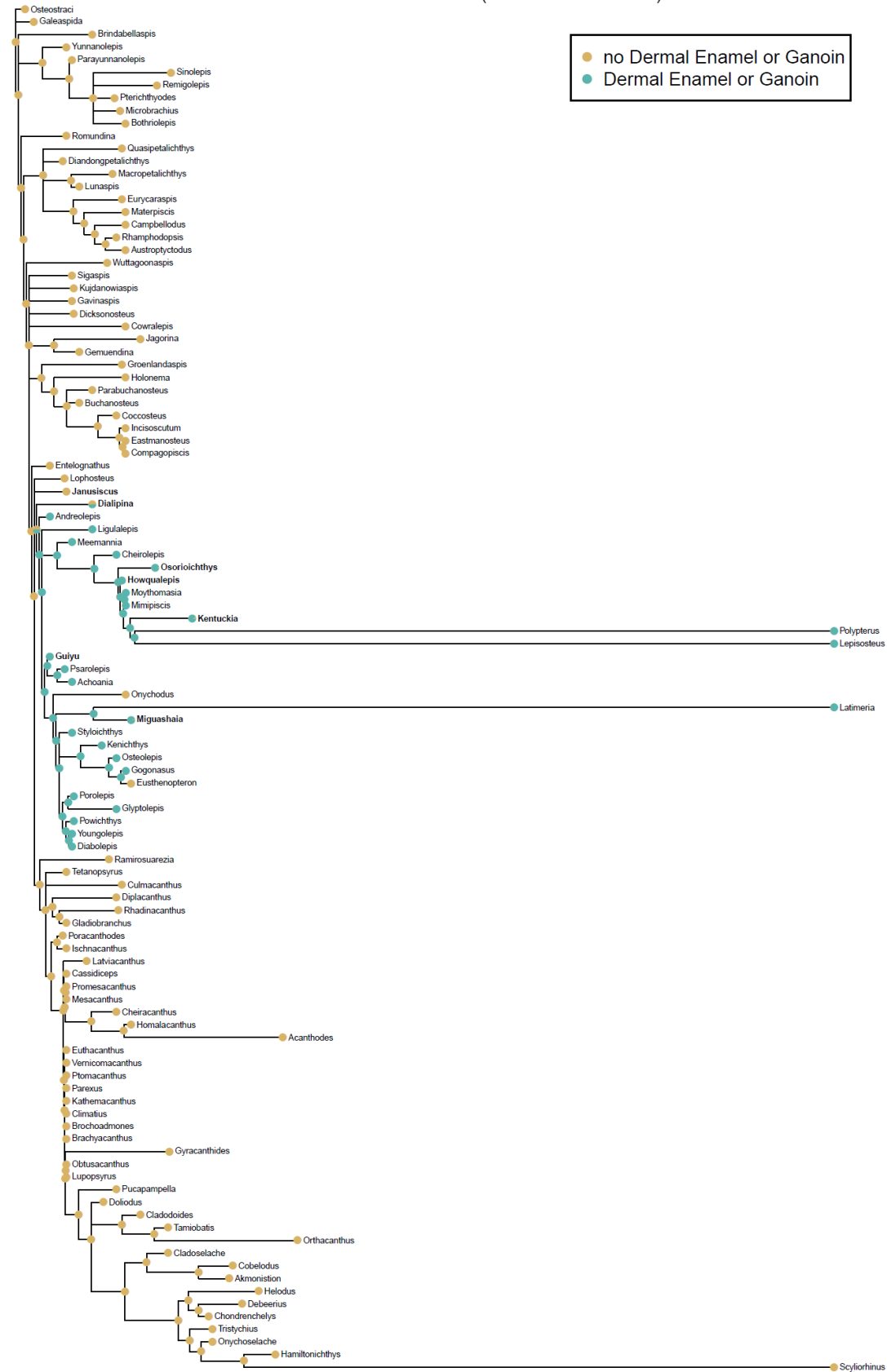
0

Dermal Enamel/Ganoin (constrained tree)



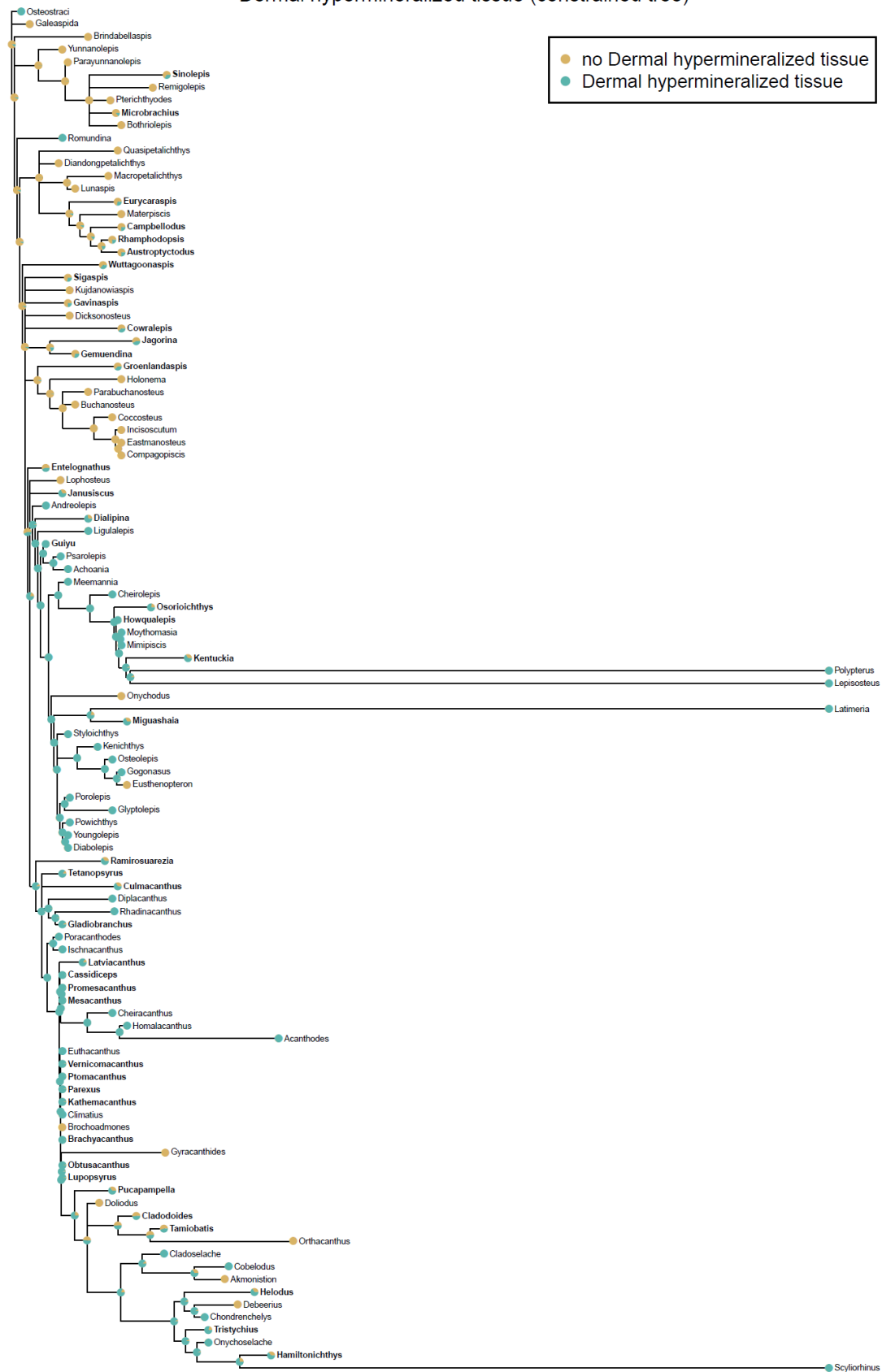
P

Dermal Enamel/Ganoin (unconstrained tree)



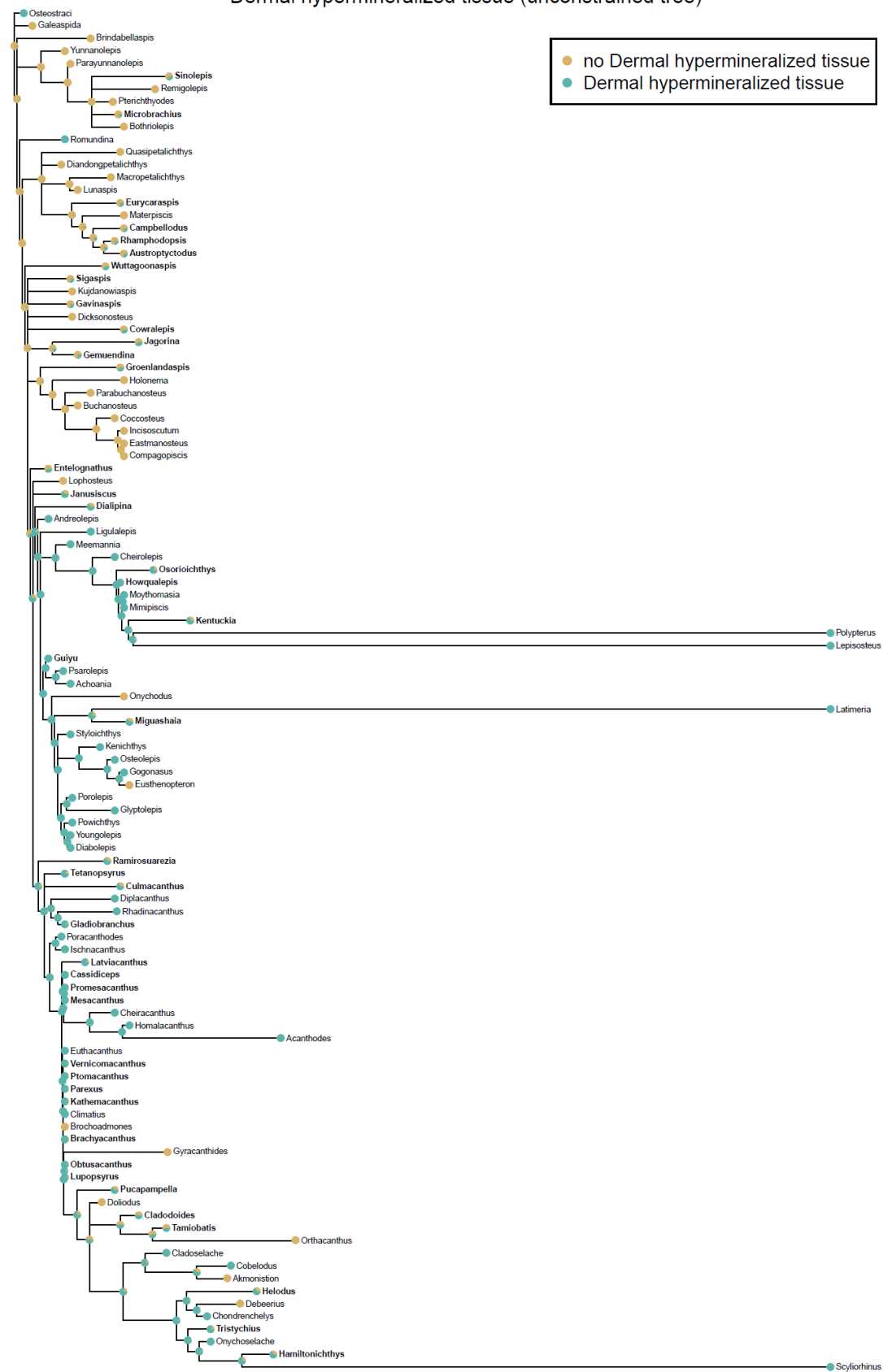
Q

Dermal hypermineralized tissue (constrained tree)



R

Dermal hypermineralized tissue (unconstrained tree)



S

overview

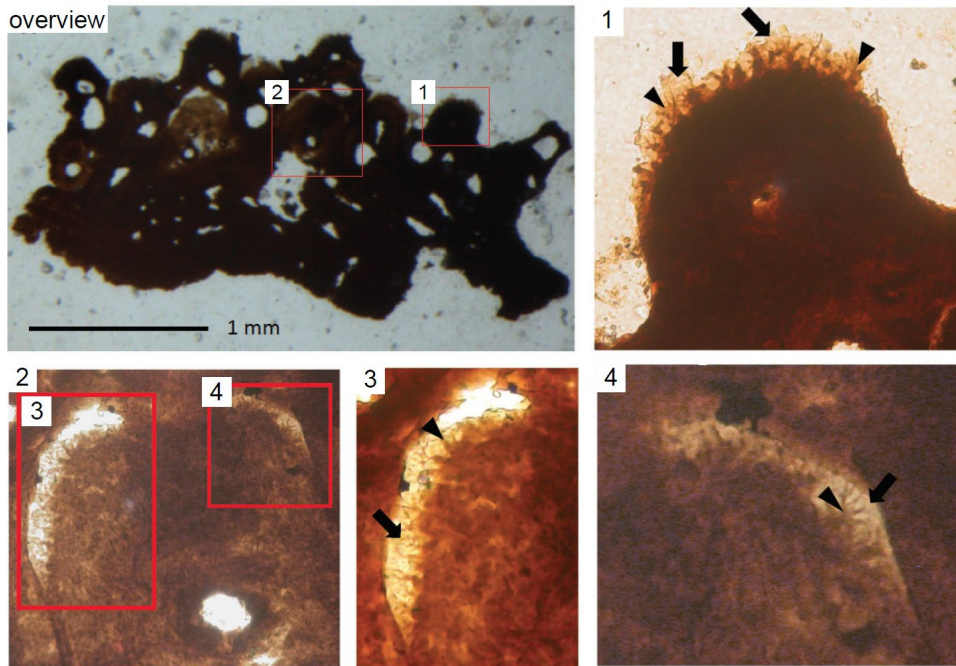


Figure S3. Reconstructing ancestral states of hypermineralized tissues, Related to Figure 5.

(A) Constrained tree used in ancestral state estimation. The GAP clade is constrained to a stem-osteichthyan affinity. Reliabilities of branches are shown at nodes as posterior probabilities.

(B) Unconstrained tree used in ancestral state estimation. The affinity of the GAP clade was unconstrained in the analysis and is resolved to a stem-sarcopterygian affinity. Reliabilities of branches are shown at nodes as posterior probabilities.

(C) Prediction of ancestral states of dental acroдин - GAP clade stem-osteichthyans, shown as pie charts.

(D) Prediction of ancestral states of dental acroдин - GAP clade unconstrained, shown as pie charts.

(E) Prediction of ancestral states of dental enameloid - GAP clade stem-osteichthyans, shown as pie charts.

(F) Prediction of ancestral states of dental enameloid - GAP clade unconstrained, shown as pie charts.

(G) Prediction of ancestral states of dermal enameloid - GAP clade stem-osteichthyans, shown as pie charts.

(H) Prediction of ancestral states of dermal enameloid - GAP clade unconstrained, shown as pie charts.

(I) Prediction of ancestral states of dental enamel - GAP clade stem-osteichthyans, shown as pie charts.

(J) Prediction of ancestral states of dental enamel - GAP clade unconstrained, shown as pie charts.

(K) Prediction of ancestral states of dermal enamel - GAP clade stem-osteichthyans, shown as pie charts.

(L) Prediction of ancestral states of dermal enamel - GAP clade unconstrained, shown as pie charts.

(M) Prediction of ancestral states of dermal ganoin - GAP clade stem-osteichthyans, shown as pie charts.

(N) Prediction of ancestral states of dermal ganoin - GAP clade unconstrained, shown as pie charts.

(O) Prediction of ancestral states of dermal enamel+ganoin - GAP clade stem-osteichthyans, shown as pie charts.

(P) Prediction of ancestral states of dermal enamel or ganoin - GAP clade unconstrained, shown as pie charts.

(Q) Prediction of ancestral states of dermal enamel, ganoin, or enameloid - GAP clade stem-osteichthyans, shown as pie charts.

(R) Prediction of ancestral states of dermal enamel, ganoin, or enameloid - GAP clade unconstrained, shown as pie charts.

(S) Cladoselache skin denticles [specimen P.9294 (NHMUK)]. Detail of boxed areas (1-4) in overview is enlarged. The dentin tubules (arrowheads), protruding through the enameloid layer (arrow), are clearly visible.

	lpq20	lpq8	lpq7	enam	scpp5	scpp7	lpq6	ambn	lpq5	lpq4	scpp3dl	scpp3cl	scpp3bl	scpp3al	odam	lpq3	scpp9	lpq2	lpq1
Coverage ¹	173	16	5	1761	6385	1768	3689	825	12	4	0	0	189	237	209	1119	69	336	2755
FPKM	54	6	2	548	2204	547	1118	262	4	2	0	0	101	106	66	338	21	102	1181
Conf ²	4	3	1	8	35	31	45	7	1	1	0	0	45	22	5	12	4	9	62

(continued)

	dmp1	scpp1	dspp1	ibsp	mepe1	mepe2	Spp1	lpq17	lpq16b	lpq16a	lpq15	lpq14	lpq13	lpq12	lpq19	lpq11	lpq18	lpq1	lpq9
Coverage ¹	33	237	33	632	513	991	1089	66	305	264	1010	79	126	951	386	1043	1201	3180	108
FPKM	12	72	11	211	193	313	326	20	125	101	341	23	38	317	117	331	383	1037	35
Conf ²	1	5	1	7	22	35	11	2	21	16	8	9	5	6	4	9	7	13	7

Table S1. Expression levels gar SCPP genes in the skin, Related to Figure 2. Relative expression levels of SCPP genes in the skin were estimated as fragments per kilobase of transcript per million mapped reads (FPKM values) (Trapnell et al., 2010). ¹Coverage shows the estimate for the absolute depth of read coverage across the whole transcript. ²Conf represents the 95% confidence interval.

	Dermal characters					Dental characters		
Taxa	Enameloid	Ganoin	Enamel	Enamel+Ganoin	Enamel+Ganoin+Enameloid	Acrodin	Enameloid	Enamel
Galeaspida	0	0	0	0	0	-	-	-
Osteostraci	1	0	0	0	1	-	-	-
Acanthodes	1	0	0	0	1	0	0/1	0/1
Achoania	0	0	1	1	1	0/1	0/1	0/1
Akmonistion	0	0	0	0	0	0	1	0
Austroptyctodus	0/1	0	0	0	0/1	0/1	0/1	0/1
Bothriolepis	0	0	0	0	0	-	-	-
Brachyacanthus	0/1	0	0	0	0/1	-	-	-
Brindabellaspis	0	0	0	0	0	0/1	0/1	0/1
Brochoadmones	0	0	0	0	0	0/1	0/1	0/1
Buchanosteus	0	0	0	0	0	0/1	0/1	0/1
Campbellodus	0/1	0	0	0	0/1	0/1	0/1	0/1
Cassidiceps	0/1	0	0	0	0/1	0/1	0/1	0/1
Cheiracanthus	1	0	0	0	1	0/1	0/1	0/1
Cheirolepis	0	1	0	1	1	1	0	0
Chondrenchelys	1	0	0	0	1	0/1	0/1	0/1
Cladodoides	0/1	0	0	0	0/1	0/1	0/1	0/1
Cladoselache	1	0	0	0	1	0	1	0
Climatius	1	0	0	0	1	0/1	0/1	0/1
Cobelodus	1	0	0	0	1	0/1	0/1	0/1
Coccosteus	0	0	0	0	0	0/1	0/1	0/1
Compagopiscis	0	0	0	0	0	0	0	0
Cowralepis	0/1	0	0	0	0/1	0/1	0/1	0/1
Culmacanthus	0/1	0	0	0	0/1	0/1	0/1	0/1
Debeerius	-	-	0	0	0	-	-	-
Diabolepis	0	0	1	1	1	0	0	1
Dialipina	0	0/1	0	0/1	0/1	0/1	0/1	0/1
Diandongpetalichthys	0	0	0	0	0	0/1	0/1	0/1
Dicksonosteus	0	0	0	0	0	0/1	0/1	0/1
Diplacanthus	1	0	0	0	1	-	-	-
Doliodus	0	0	0	0	0	0/1	0/1	0/1
Eastmanosteus	0	0	0	0	0	0/1	0/1	0/1
Entelognathus	0/1	0	0	0	0/1	-	-	-
Eurycaraspis	0/1	0	0	0	0/1	0/1	0/1	0/1
Eusthenopteron	0	0	0	0	0	0	0	1
Euthacanthus	1	0	0	0	1	-	-	-
Gavinaspis	0/1	0	0	0	0/1	0/1	0/1	0/1
Gemuendina	0/1	0	0	0	0/1	0/1	0/1	0/1
Gladiobranchus,	0/1	0	0	0	0/1	-	-	-
Glyptolepis	0	0	1	1	1	0	0	1
Gogonaspis	0	0	1	1	1	0	0	1
Groenlandaspis	0/1	0	0	0	0/1	0/1	0/1	0/1
Guiyu	0	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Gyracanthides	0	0	0	0	0	-	-	-
Scyllorhinus	1	0	0	0	1	0	1	0
Hamiltonichthys	0/1	0	0	0	0/1	0	1	0
Helodus	0/1	0	0	0	0/1	0	1	0
Holonema	0	0	0	0	0	0	0	0
Homalacanthus	1	0	0	0	1	-	-	-
Howqualepis	0	0/1	0	0/1	0/1	0/1	0/1	0/1
Incisoscutum	0	0	0	0	0	0/1	0/1	0/1
Ischnacanthus	1	0	0	0	1	0	1	0
Jagorina	0/1	0	0	0	0/1	0/1	0/1	0/1
Janusiscus	0/1	0	0/1	0/1	0/1	0/1	0/1	0/1

Kathemacanthus	0/1	0	0	0	0/1	-	-	-
Kenichthys	0	0	1	1	1	0/1	0/1	0/1
Kentuckia	0	0/1	0	0/1	0/1	0/1	0/1	0/1
Kujdanowiaspis	0	0	0	0	0	0/1	0/1	0/1
Latviacanthus	0/1	0	0	0	0/1	0	0	0
Ligulalepis	0	1	0	1	1	1	0	0
Lophosteus	0	0	0	0	0	0	0	0
Lunaspis	0	0	0	0	0	0/1	0/1	0/1
Lupopsyrus	0/1	0/1	0	0/1	0/1	-	-	-
Macropetalichthys	0	0	0	0	0	0/1	0/1	0/1
Materpiscis	0	0	0	0	0	0/1	0/1	0/1
Meemannia	0	0	1	1	1	0/1	0/1	0/1
Mesacanthus	0/1	0	0	0	0/1	-	-	-
Microbrachius	0/1	0	0	0	0/1	0/1	0/1	0/1
Miguashaia	0/1	0/1	0	0/1	0/1	0	0	1
Lepisosteus	0	1	0	1	1	1	0	1
Polypterus	0	1	0	1	1	1	0	1
Mimipiscis	0	1	0	1	1	1	0	0
Moythomasia	0	1	0	1	1	1	0	0
Obtusacanthus	0/1	0	0	0	0/1	-	-	-
Onychodus	0	0	0	0	0	0	0	1
Onychoselache	1	0	0	0	1	0	1	0
Orthacanthus	0	0	0	0	0	0	1	0
Osorioichthys	0	0/1	0	0/1	0/1	1	0	0
Osteolepis	0	0	1	1	1	0/1	0/1	0/1
Parabuchanosteus	0	0	0	0	0	0/1	0/1	0/1
Parayunnanolepis	0	0	0	0	0	0/1	0/1	0/1
Parexus	0/1	0	0	0	0/1	0	0	0
Poracanthodes	1	0	0	0	1	0/1	0/1	0/1
Porolepis	0	0	1	1	1	0	0	1
Powichthys	0	0	1	1	1	0	0	1
Promesacanthus	0/1	0	0	0	0/1	-	-	-
Psarolepis	0	0	1	1	1	0	0	0
Pterichthyodes	0	0	0	0	0	0/1	0/1	0/1
Ptomacanthus	0/1	0	0	0	0/1	0/1	0/1	0/1
Pucapampella	0/1	0	0	0	0/1	0/1	0/1	0/1
Quasipetalichthys	0	0	0	0	0	0/1	0/1	0/1
Ramiroisuaresia	0/1	0	0	0	0/1	0/1	0/1	0/1
Remigolepis	0	0	0	0	0	0/1	0/1	0/1
Rhadinacanthus	1	0	0	0	1	-	-	-
Rhamphodopsis	0/1	0	0	0	0/1	0	0	0
Romundina	1	0	0	0	1	0/1	0/1	0/1
Sigaspis	0/1	0	0	0	0/1	0/1	0/1	0/1
Sinolepis	0/1	0	0	0	0/1	0/1	0/1	0/1
Styloichthys	0	0	1	1	1	0/1	0/1	0/1
Latimeria	0	0/1	1	1	1	0	0	1
Tamiobatis	0/1	0	0	0	0/1	0/1	0/1	0/1
Tetanopsyrus	0/1	0	0	0	0/1	-	-	-
Tristychius	0/1	0	0	0	0/1	0/1	0/1	0/1
Vernicomacanthus	0/1	0	0	0	0/1	0	0	0
Wuttagoonaspis	0/1	0	0	0	0/1	0/1	0/1	0/1
Youngolepis	0	0	1	1	1	0	0	1
Yunnanolepis	0	0	0	0	0	0/1	0/1	0/1
Andreolepis	0	0	1	1	1	0	0	0

Table S2. Presence or absence of enameloid, ganoin, enamel, enamel or ganoin, enamel, ganoin, or enameloid in the dermal skeleton, and acrodin, enameloid, and enamel on teeth, Related to Figure 5. 0, 1, and 0/1 represent the absence, presence, and unknown status of the tissue, respectively. “-” in dental characters represents the absence of teeth.

Taxa	Minimum age (Mya)	Reference
Galeaspida	432.6	
Osteostraci	437.4	
Acanthodes	298	King et al. 2016
Achoania	412	King et al. 2016
Akmonistion	327	King et al. 2016
Austroptyctodus	383	King et al. 2016
Bothriolepis	383	King et al. 2016
Brachyacanthus	415	King et al. 2016
Brindabellaspis	401	King et al. 2016
Brochoadmones	415	King et al. 2016
Buchanosteus	408	King et al. 2016
Campbellodus	383	King et al. 2016
Cassidiceps	415	King et al. 2016
Cheiracanthus	388	King et al. 2016
Cheirolepis	388	King et al. 2016
Chondrenchelys	338	King et al. 2016
Cladodoides	375	King et al. 2016
Cladoselache	360	King et al. 2016
Climatius	415	King et al. 2016
Cobelodus	325	King et al. 2016
Coccosteus	388	King et al. 2016
Compagopiscis	383	King et al. 2016
Cowralepis	383	King et al. 2016
Culmacanthus	385	King et al. 2016
Debeerius	320	King et al. 2016
Diabolepis	412	King et al. 2016
Dialipina	401	King et al. 2016
Dandongpetalichthys	417	King et al. 2016
Dicksonosteus	411	King et al. 2016
Diplacanthus	388	King et al. 2016
Doliodus	395	King et al. 2016
Eastmanosteus	383	King et al. 2016
Entelognathus	424	King et al. 2016
Eurycaraspis	385	King et al. 2016
Eusthenopteron	380	King et al. 2016
Euthacanthus	415	King et al. 2016
Gavinaspis	412	King et al. 2016
Gemuendina	408	King et al. 2016
Gladiobranchus	415	King et al. 2016
Glyptolepis	388	King et al. 2016
Gogonaspis	383	King et al. 2016
Groenlandaspis	385	King et al. 2016
Guiyu	424	King et al. 2016
Gyracanthides	359.3	Warren et al. 2000
Scylliorhinus	0	
Hamiltonichthys	302	King et al. 2016
Helodus	311	King et al. 2016
Holonema	383	King et al. 2016
Homalacanthus	380	King et al. 2016
Howqualepis	385	King et al. 2016
Incisoscutum	383	King et al. 2016
Ischnacanthus	415	King et al. 2016
Jagorina	375	King et al. 2016
Janusiscus	415	King et al. 2016
Kathemacanthus	415	King et al. 2016
Kenichthys	396	King et al. 2016
Kentuckia	347	King et al. 2016
Kujdanowiaspis	411	King et al. 2016
Latviacanthus	404	King et al. 2016
Ligulalepis	401	King et al. 2016
Lophosteus	416	Cunningham et al. 2012
Lunaspis	408	King et al. 2016
Lupopsyrus	415	King et al. 2016
Macropetalichthys	390	King et al. 2016
Materpiscis	383	King et al. 2016
Meemannia	412	King et al. 2016
Mesacanthus	415	King et al. 2016
Microbrachius	386	King et al. 2016
Miguashaia	380	King et al. 2016
Lepisosteus	0	
Polypterus	0	
Mimipiscis	383	King et al. 2016
Moythomasia	383	King et al. 2016

Obtusacanthus	415	King et al. 2016
Onychodus	383	King et al. 2016
Onychoselache	336	King et al. 2016
Orthacanthus	290	King et al. 2016
Osorioichthys	367	King et al. 2016
Osteolepis	388	King et al. 2016
Parabuchanosteus	401	King et al. 2016
Parayunnanolepis	412	King et al. 2016
Parexus	415	King et al. 2016
Poracanthodes	417	King et al. 2016
Porolepis	411	King et al. 2016
Powichthys	411	King et al. 2016
Promesacanthus	415	King et al. 2016
Psarolepis	416	King et al. 2016
Andreolepis	424	Chen et al. 2016
Pterichthyodes	389	King et al. 2016
Ptomacanthus	415	King et al. 2016
Pucapampella	388	King et al. 2016
Quasipetalichthys	385	King et al. 2016
Ramirosuarezia	392	King et al. 2016
Remigolepis	366	King et al. 2016
Rhadinacanthus	386.9	Lukševičs et al. 2010
Rhamphodopsis	388	King et al. 2016
Romundina	415	King et al. 2016
Sigaspis	412	King et al. 2016
Sinolepis	358.5	Zhu et al. 2000
Styloichthys	412	King et al. 2016
Latimeria	0	
Tamiodont	360	King et al. 2016
Tetanopsyrus	415	King et al. 2016
Tristychius	336	King et al. 2016
Vernicomacanthus	415	King et al. 2016
Wuttagoonaspis	393	King et al. 2016
Youngolepis	412	King et al. 2016
Yunnanolepis	415	King et al. 2016

Table S3. Tip ages used for the ancestral state estimation, Related to Figure 5.

Transparent Methods

Bioinformatic analyses

We searched for SCPP genes in RNA-seq datasets (GenBank accession numbers: SRX796494 and SRX1016233-SRX1016241 for lungfish, SRX796491 and SRX1386644- SRX1386647 for bichir, and SRX424533 and SRX424534 for sturgeon) by tblstn (<http://www.ncbi.nlm.nih.gov/>) (Altschul et al., 1990) using amino acid sequences of gar orthologs as queries (Kawasaki et al., 2017).

Relative expression levels of gar SCPP genes were estimated as FPKM values (Trapnell et al., 2010), calculated for a dataset of gar skin (GenBank accession number, SRP042013) (Braasch et al., 2016) using Galaxy (<https://usegalaxy.org/>) (Afgan et al., 2016). The dataset was retrieved from the EBI SRA database, trimmed using Trimmatic (Bolger et al., 2014), aligned with the gar genome sequence (LepOcu1) using TopHat (Kim et al., 2013). FPKM values and confidence intervals were calculated using Cufflinks (Trapnell et al., 2010) based on BAM files obtained by the TopHat analysis. All default conditions were used in Galaxy analysis, except additional options in Trimmomatic (ILLUMINACLIP and MINLEN=50) and Cufflinks (multi-read correction). Genomic coordinates of SCPP genes were determined using Splign (<http://www.ncbi.nlm.gov/>) (Kapustin et al., 2008), and used for the Cufflinks analysis.

Molecular analysis

All animals used in our study (gar, 16-55 cm in total length; zebrafish, 3.0 cm in total length; bichir, 20 cm in total length; and lungfish, 20 cm in total length; sex not determined for these animals) were sacrificed according to the guidelines issued by the Ministry of Justice in Japan. SCPP genes identified in bichir and lungfish RNA-seq datasets were confirmed by PCR using cDNA libraries made from a tooth plate (lungfish) and tooth germs (bichirs), as described (Braasch et al., 2016). These libraries were made using SMART cDNA library construction kit (Takara Bio), and PCR products were size fractionated by agarose gel electrophoresis, purified from the agarose gel using the FastGene Gel/PCR Extraction Kit (Nippon Genetics), and ligated into T-vector pMD20 (Takara Bio). The ligation mix was then used to transform *E.*

coli HSTo8 Premium Competent Cells (Takara Bio). Exon-intron borders were also determined by PCR using genomic DNA as the substrate. For expression analysis of bichir *scpp5*, total RNA molecules were isolated using RNAiso Plus (Takara Bio) and cDNA was synthesized using the PrimerScript II 1st strand cDNA synthesis kit (Takara bio). Primer sequences used for RT-PCR are as follows: 5'-GAGACTTGCGATCTTCTCTTG-3' and 5'-GTGAATTGACCTGAGGCAGGA-3' for *scpp5*; and 5'-CACAGTTTGCCAGATGGTCC-3' and 5'-CACCACCAATTGCCTTGCTC-3' for *gapdh*.

ISH analysis

For ISH analysis, jaws and skin of gar and zebrafish were fixed with neutralized 4% paraformaldehyde, decalcified with Morse's solution (10% w/v sodium citrate and 22.5% v/v formic acid), dehydrated through a graded ethanol series and xylene, embedded in paraffin, sectioned in the coronal plane at 4 µm in thickness, and mounted on glass slides. These glass slides were deparaffined, treated with Proteinase K (20 mg/ml) for 10 min, and used for hybridization (50% formamide, 10 mM Tris pH7.6, 1xDenhart's solution, 5% dextran sulfate, 600 mM NaCl, 0.25% SDS, 1 mM EDTA, and 100µg/ml *Escherichia coli* tRNA) at 70°C (Nakatomi et al., 2006). A specific portion of *scpp5*, *ambn*, and *enam* were amplified from the cDNA libraries (primer sequences: 5'-GTTGGTGCTACAGCAGGAAGT-3' and 5'-GTTGTGCTTCCCTGAACTG-3' for gar *ambn*; 5'-AAGGCCTCAGCTTCGTCCAG-3' and 5'-ACTCCTTCTCGTTGACTTCGT-3' for gar *enam*; 5'-TATTCTGAGGAGTCACCAA-3' and 5'-ATTCTGACTTGGCTGGACG-3' for gar *scpp5*; 5'-ACTTCATCAACAGGTGCCCCAATC-3' and 5'-TGAAAGCTCCGTGACCTGAATCT-3' for zebrafish *ambn*; and 5'-CTGCCCCTGACAGTGGCAGTAATG-3' and 5'-CATCAGGCCCAACAACAGTGGTGT-3' for zebrafish *enam*), cloned using the pGEM-T Easy Vector (Promega), and labeled with digoxigenin using the DIG RNA Labeling Kit (Roche), and used for hybridization. After hybridization, glass slides were washed with 2xSSC. Hybridization signals were detected using Anti-Digoxigenin-AP, Fab fragments (Roche) and NBT/BCIP Solution (Roche), as specified.

IHC analysis

For IHC analysis, jaws and scales were fixed (4% paraformaldehyde-0.2% glutaraldehyde, 0.05M HEPES buffer pH7.4), dehydrated, and embedded in LR-White resin (LR-W, London Resin), and processed for the Protein A-gold (PAG) method (Sasagawa et al., 2012). For light microscopic analysis, semi-thin sections were made from LR-W resin block, mounted on glass slides, masked with 5% goat serum in phosphate buffered saline (PBS), and reacted with the antibody diluted 1:100 or 1:200 in PBS containing 0.5% bovine serum albumin (PBS-BSA) at 4°C. These sections were washed with PBS, incubated with the PAG conjugate (gold nanoparticles 5nm, BBI) diluted 1:100 with PBS-BSA, washed with PBS, treated with the silver enhancer solution (50 mM citrate buffer, 0.85% hydroquinone, 0.1% maleic acid, 0.11% silver lactate, and 1% acacia powder) (Uchida et al., 1991) for 10 min in a dark box, and rinsed with water. These sections were then immersed in photographic fixatives, and stained with fast red or toluidine blue (Sasagawa et al., 2012).

For TEM analysis, ultrathin sections obtained from LR-W resin block were mounted on nickel grids, floated on a drop of 1% goat serum and then the antibody diluted 1:200-1:400 in PBS-BSA. These sections were subsequently washed with PBS-BSA, treated with 1% goat serum and then with the PAG conjugate diluted 1:10 in PBS-BSA. These sections were stained with platinum blue and lead citrate (additionally stained with phosphotungstic acid for some samples), and examined using TEM (JEM-1010, JEOL) (Sasagawa et al., 2016). In some sections, immunoreactions were enhanced using the silver enhancer solution (Uchida et al., 1991) or the Silver Enhancer Kit (Kirkegaard & Perry Laboratories). Polyclonal antibodies to gar Scpp5 were raised against YRQQPQQN (SIGMA-ALDRICH).

Phylogenetic analysis

We augmented an existing phylogenetic dataset (Qiao et al., 2016) with codings for *Scyliorhinus*, *Lepisosteus*, *Polypterus*, and *Latimeria*, representing the three major living clades of jawed vertebrates. We also coded *Andreolepis*, a putative stem-osteichthyan (Qu et al., 2015), and coded *Ligulalepis* as

present for dental acrodin (Schultze, 2016; Schultze, 2018). Morphological data were analyzed using the Mkv+G model in MrBayes 3.2.7 (Ronquist et al., 2012). We constrained the positions of the extant taxa to mitigate long branch effects: *Scyliorhinus* with *Helodus*, *Chondrenchelys*, *Debeerius*, *Tristychius*, *Hamiltonichthys*, *Onychoselache* (Qiao et al., 2016); *Latimeria* with *Miguashaia* (Arratia and Schultze, 2015); and *Polypterus*, *Lepisosteus*, *Kentuckia* with *Moythomasia*, *Mimipiscis* (Giles et al., 2017). We conducted two phylogenetic analyses of this dataset, one in which the GAP clade were constrained to be stem-osteichthyans (King et al., 2017; Lu et al., 2017), and the other in which these taxa were topologically unconstrained, yielding a stem-sarcopterygian affinity (Lu et al., 2016; Qiao et al., 2016; Choo et al., 2017). Each analysis used four independent runs of four chains over 10,000,000 generations, sampling every 10,000 generations, with 25% of burnin. Convergence was assessed using Tracer 1.7 (Rambaut et al., 2018). Results were summarized using majority rule consensus trees (Figures S3A and S3B).

Ancestral state estimation

We compiled data on the distribution of five dermal (enameloid, ganoin, enamel, ganoin/enamel, hypermineralized tissue) and three dental (acrodin, enameloid, enamel) skeletal characters (Table S2); we confirmed the presence of dental enameloid in *Cladoselache* (Figure S3S).

Branch lengths were estimated using the function *timePaleoPhy* in the R package (<https://www.R-project.org/>) *paleotree* (Bapst, 2012) using the ‘equal’ method (Brusatte et al., 2008), with the root age increased by 5 million years. Tip ages are from King et al. (King et al., 2017) (Table S3) but the root (Tinn and Märss, 2018), and the crown clades of chondrichthyans, sarcopterygians, actinopterygians, osteichthyans, and gnathostomes (Benton et al., 2015) were calibrated to be minimally as old as oldest fossil representative. Using these time-scaled topologies, we estimated ancestral states using Stochastic Character Mapping (Huelsenbeck et al., 2003) in the R package *phytools* (Revell, 2012). Each character was coded as a binary presence or absence state for all taxa; uncertain tip states were assigned an equal prior probability of 0.5. Taxa were excluded from an analysis when the character was

inapplicable. By running all separate analyses for each character, there is zero modelled co-variance between each character. Stochastic character mapping was run using the *make.simmap* function (Bollback, 2006) in *phytools* but with a single Q matrix for all simulations under the 'all rates different' and a naïve equal prior probability for absence or presence at the root. Each character model ran for 1000 simulations and each iteration used the same 'empirical' Q matrix with the highest likelihood transition probabilities. Results were summarized using the *describe.simmap* function and the posterior probabilities of node and tip states were plotting on each tree.

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